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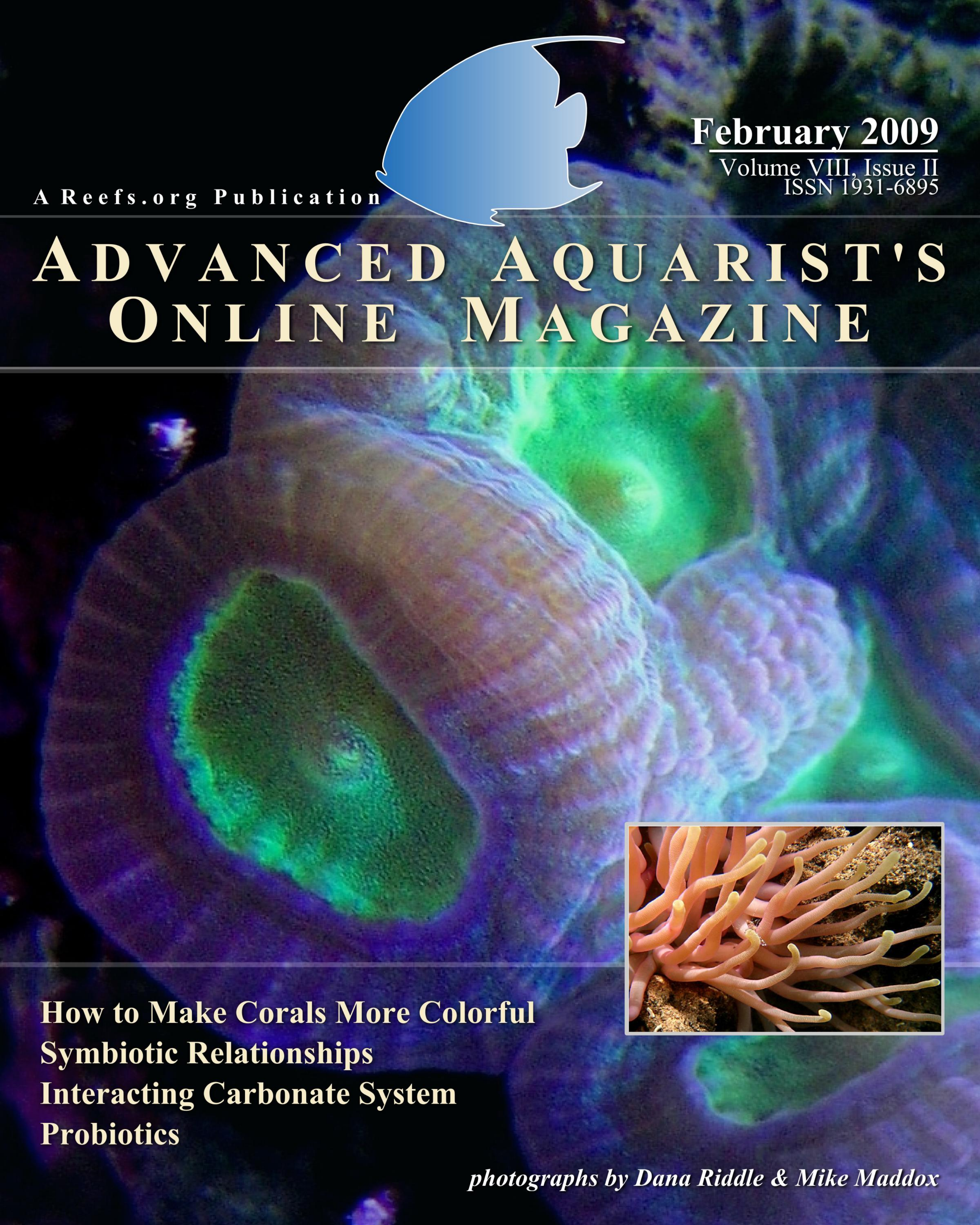

How to Make Corals More Colorful
Symbiotic Relationships
Interacting Carbonate System
Probiotics

photographs by Dana Riddle & Mike Maddox

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Caulastrea furcata coral (main) and *Condylactys gigantae* anemone (inset).

Photographs by Dana Riddle and Mike Maddox

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Aquatic & Terrestrial Research Team. While Probiotics continue to grow in popularity, it is important to look at the actual organisms found in culture.

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EDITORIAL

FEBRUARY 2009

By Terry Siegel

Terry discusses upcoming featured aquariums, new features for the magazine, and some exciting news about the Reefs.org website.

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Reef keepers, along with research by scientists, keep refining our understanding of the nuances of the biochemical elements of our hobby. And, though there are still some animals that have yet to be successfully kept in our reef aquaria – *Dendronepthya* sp., for example – we have become pretty good at it. In fact, the challenge of reef keeping may have become a wee bit routine. So, in the next issue (March 2009) of *Advanced Aquarist*, we will publish an article on maintaining a temperate aquarium.

Some of you reef keepers may be thinking, why bother? Let me show you some pictures from a coming article in March to answer that question.

NEW MAGAZINE FEATURES

Two new features that we're rolling out this month on the website are a new commenting system and a better social bookmarking link. A screenshot of both can be seen below.

Our new comments system is powered by IntenseDebate (a Wordpress/Automatix owned application) and has been used on many high-profile websites such as President Barak Obama's Change.gov. Some of the features include:

- Threaded Questions
- RSS feeds for both individual comments and all comments
- Receive notice via email that someone's made a comment

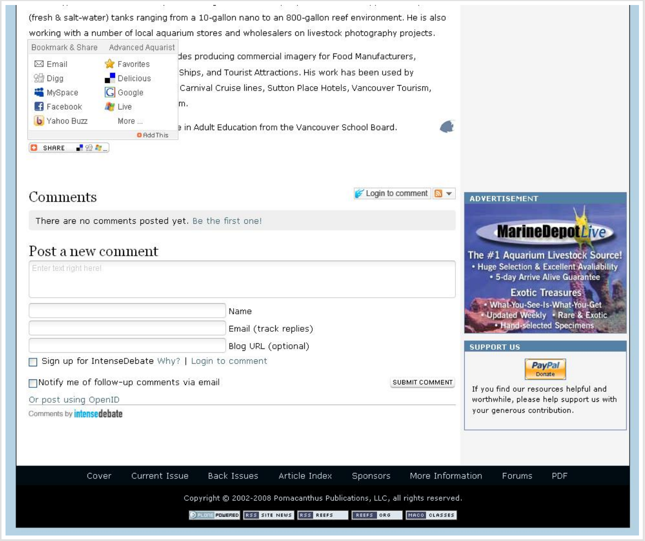
And if you choose to register with IntenseDebate, you can:

- Register with your OpenID or the normal signup procedure
- Get/Post comments by email
- Use Gravatars
- Have commenter profiles
- Reputation points and comment voting
- And more!

We've wanted to implement comments for quite some time now and we encourage our users to comment away!

COMING NEXT MONTH AT REEFS.ORG

Reefs.org is expected to roll out a much more powerful bulletin board software next month, with a lot of improvements in performance and functionality. Stay tuned!



(fresh & salt-water) tanks ranging from a 10-gallon nano to an 800-gallon reef environment. He is also working with a number of local aquarium stores and wholesalers on livestock photography projects.

des producing commercial imagery for Food Manufacturers, Ships, and Tourist Attractions. His work has been used by Carnival Cruise lines, Sutton Place Hotels, Vancouver Tourism, m.

e in Adult Education from the Vancouver School Board.

Comments

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New commenting system and a more useful bookmarking app are implemented at the end of articles.

FEATURE ARTICLE

HOW TO MAKE CORALS MORE COLORFUL, PART TWO: NEW INFORMATION! GREEN FLUORESCENT PIGMENTS, PIGMENT CLADES, AND PHOTOCONVERSION FROM GREEN TO ORANGE/RED

By Dana Riddle

This month, we'll look at the most populous coral pigment category: Green Fluorescent Proteins (or GFPs). In addition, we'll examine how light intensity and spectral qualities affect GFPs.

Published February 2009, Advanced Aquarist's Online Magazine.

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Last time, in Part 1 of this series, we reviewed technical terms useful in the understanding of coral coloration. We learned there are many different categories of colorful or fluorescent pigments, and these pigments can be further divided into classes (or clades) where ancestral origins are important in classification tools.

The most common fluorescent pigments are green. Note the granular appearance of the green fluorescent chromophores within this coral's tissues. Photo by the author.

This month, we'll look at the most populous coral pigment category: Green Fluorescent Proteins (or GFPs). In addition, we'll examine how light intensity and spectral qualities affect GFPs. It is important to note that some GFPs can be converted by light to other colors (especially orange or red fluorescence). An examination of photoconversion will be part of this month's installment.

To make things easier if you're not familiar with the terminology, I'll present the glossary once more:

GLOSSARY

Absorbance:
Ability of a solution or layer of a substance to retain light without reflection or transmission.

Absorption:
The process in which incident radiation is retained without reflection or transmission.

Brightness:
The intensity of a fluorescent emission. Extinction coefficient times Quantum Yield = Brightness.

Clade:
For our purposes in this article, a grouping of pigments based on similar features inherited from a common ancestor. Pigments from corals includes Clades A, B, C, and D. Clades can refer to living organisms as well (clades of *Symbiodinium* - zooxanthellae - are a good example.)

Chromophore:

The colorful portion of a pigment molecule. In some cases, chromophore refers to a granular packet containing many pigment molecules.

Chromoprotein pigment:

A non-fluorescent but colorful pigment. These pigments appear colorful because they reflect light. For example, a chromoprotein with a maximum absorption at 580nm might appear purple because it preferentially reflects blue and red wavelengths.

Chromo-Red Pigment:

A newly described type of pigment possessing characteristics of both chromoproteins and Ds-Red fluorescent proteins. Peak fluorescence is at 609nm (super red).

Cyan Fluorescent Protein (CFP):

Blue-green pigments with fluorescent emissions in the range of ~477-500nm. Cyan and green pigments share a similar chromophore structure. Cyan pigments are expressed at lower light levels than green, red or non-fluorescent pigments.

Emission:

That light which is fluoresced by a fluorescent pigment.

Extinction Coefficient:

The quantity of light absorbed by a protein under a specific set of circumstances.

Excitation:

That light absorbed by a fluorescent pigment. Some of the excitation light is fluoresced or emitted at a less energetic wavelength (color).

Ds-Red type pigment:

A type of red fluorescent pigment with a single primary emission bandwidth at 574-620nm. Originally found in the false coral *Discosoma.*

Fluorescence:

Absorption of radiation at one wavelength (or color) and *emission* at another wavelength (color). Absorption is also called *excitation.* Fluorescence ends very soon after the excitation source is removed (on the order of ~2-3 nanoseconds: Salih and Cox, 2006).

Green Fluorescent Protein (GFP):

Fluorescent pigments with emissions of 500-525nm.

'Hula Twist':

A bending of a pigment molecule resulting in a change of apparent color. Molecular bonds are not broken; therefore the pigment can shift back and forth, with movements reminiscent of a hula dancer.

Kaede-type pigment:

A type of red fluorescent pigment with a characteristic primary emission at ~574-580nm *and* a secondary (shoulder) emission at ~630nm. Originally found in the stony coral *Trachyphyllia geoffroyi*, but common in corals of suborder Faviina.

Kindling Protein:

A protein capable of being converted from a non-fluorescent chromoprotein to a fluorescent protein. Sometimes called a 'Kindling Fluorescent Protein', or KFP.

Quantum Yield:

Amount of that energy absorbed which is fluoresced. If 100 photons are absorbed, and 50 are fluoresced, the quantum yield is 0.50.

Photobleaching:

Some pigments, such as *Dronpa*, loss fluorescence if exposed to strong light (in this case, initially appearing green and bleaching to a non-fluorescent state when exposed to blue-green light). Photobleaching can obviously cause drastic changes in apparent fluorescence. In cases where multiple pigments are involved, the loss of fluorescence (or energy transfer from a donor pigment to an acceptor pigment) could also result in dramatic shifts in apparent color.

Photoconversion:

A rearrangement of the chemical structure of a colorful protein by light. Depending upon the protein, photoconversion can increase or decrease fluorescence (in processes called photoactivation and photobleaching, respectively). Photoconversion can break proteins' molecular bonds (as with *Kaede* and *Eos* fluorescent pigments) resulting in an irreversible color shift, or the molecule can be 'twisted' by light energy (a 'hula twist') where coloration reversal are possible depending upon the quality or quantity of light available. This process is known as photoswitching).

Red Fluorescent Protein (RFP):

Those pigments with an emission of ~570nm and above. Includes Ds-Red, Kaede and Chromo-Red pigments.

Stokes Shift:

The difference in the maximum wavelength of fluorescent pigment excitation light and the maximum wavelength of the fluoresced light (emission). For example, a pigment with an excitation wavelength of 508nm and an emission wavelength of 535nm would have a Stokes Shift of 27nm.

Threshold or Coloration Threshold:

The point at which pigment production is sufficient to make its fluorescence (or in the case of non-fluorescent chromoproteins, it absorption) visually apparent. The term threshold generally refers pigment production, although, in some cases, it could apply to a light level where a pigment disappears (as in the cases of photobleaching, or photoconversion).

Yellow Fluorescent Protein:

An uncommon group of fluorescent proteins with emissions in the 525-570 nm range (Alieva et al., 2008).

TYPES OF PIGMENTS

There are at least 9 described types of coral pigments. Note: Pigment types, such as green or red *might* not be structurally similar to another green or red pigment in a different clade - see below). These include:

1. Cyan Fluorescent Proteins (CFP) - Cyan pigments are blue-green pigments with a maximum emission of up to ~500 nm. The chromophore structure of a cyan pigment is very similar to that of a green fluorescent pigment.
2. Green Fluorescent Proteins (GFP) - This group, by far, is the most numerous of the fluorescent proteins. The structure of green fluorescent chromophores is very similar to that of cyan fluorescent chromophores.
3. Yellow Fluorescent Proteins (YFP) - An unusual type of fluorescent protein with maximum emission in the yellow portion of the spectrum. Rare in its biological distribution, YFP is found in a zoanthid and some specimens of the stony coral *Agaricia*. Personally, I've noted yellow fluorescence in a very few stony corals (*Porites* specimens) here in Hawaii while on night dives using specialized equipment to observe such colorations (see www.nightsea.com for details on this equipment).
4. Orange Fluorescent Protein (OFP) - I've included this protein 'type' in an attempt to avoid confusion. OFP is used to describe a pigment found in stony coral *Lobophyllia hemprichii* and its name suggests a rather unique sort of protein. In fact, OFP is simply a variant of the Kaede-type fluorescent proteins.
5. Red Fluorescent Proteins (RFP) - A group of proteins including several different subtypes (Kaede, Ds-Red and Chromo-Red). Typically, fluorescent emission is in the range of ~580 nm to slightly over 600 nm.
6. Dronpa - A green fluorescent protein that loses fluorescent when exposed to blue-green light (~490 nm) but returns when irradiated with violet light at ~400 nm.
7. Kindling Proteins - A protein (notably from the anemone *Anemonia sculata*) that changes from a non-fluorescent pigment to one demonstrating fluorescence. This change is switchable/reversible and its state depends upon the spectral quality of light striking it.

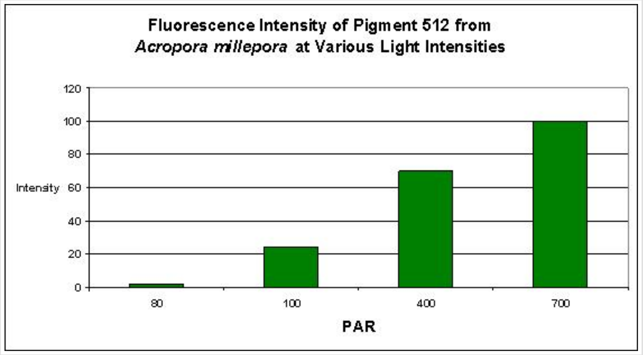

Figure 1. Light intensity and its effects on fluorescent intensity. Light was generated by a 13,000 K metal halide lamp. After D'Angelo et al., 2008.

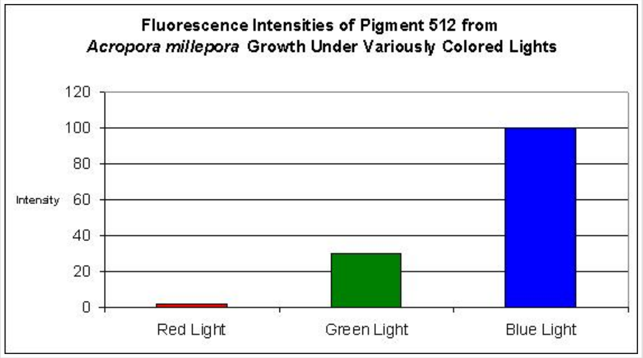

Figure 2. Effects of light color on Acropora millepora's fluorescent Pigment 512. Light intensity was 200 µmol·m²·sec in each of the treatments. After D'Angelo et al., 2008.

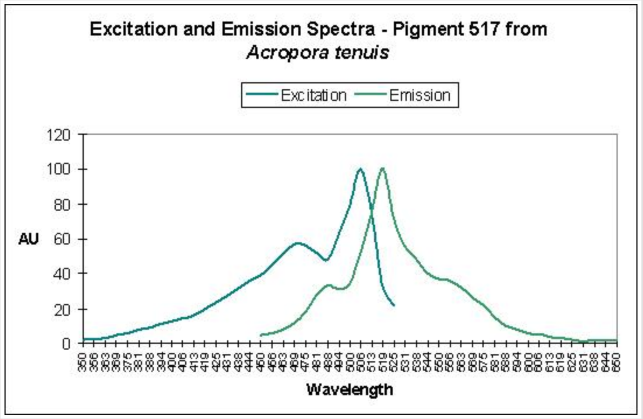

Figure 3. A case of possible photoconversion as shown by Papina et al., 2002.

8. Chromo-Red Proteins - A new classification (Alieva et al., 2008) of a single fluorescent pigment found in the stony coral *Echinophyllia*. This chromo-red pigment has qualities of a non-fluorescent chromoprotein, but fluoresces at a maximum of 609 nm.
9. Chromoproteins (CP) - This group of pigments is non-fluorescent, or has minimal fluorescence (where the quantum yield is essentially zero). Instead of relying upon fluorescence for coloration, these pigments instead absorb light most strongly in a relatively narrow portion of the visible spectrum. Most coral chromoproteins absorb light maximally at 560-593 nm. There are reports of anemones absorbing light at a maximum wavelength of 610nm, and a couple of reports of stony corals absorbing wavelengths in the 480-500nm range. Some chromoproteins are very similar in structure to the fluorescent Ds-Red proteins. In fact, genetic engineers have found that a single amino acid substitution in a protein can make the difference between non-fluorescence and fluorescence. Chromoproteins do not get much attention by researchers (relative to that of fluorescence proteins) and there are only about 40 described.

EFFECTS OF LIGHT INTENSITY AND SPECTRAL QUALITY ON GFPS

The following information is derived mostly from the work of D'Angelo et al. (2008). It offers fascinating insights into some of the factors required to promote production of fluorescent pigments in corals. The primary investigator in the D'Angelo experiments was Dr. Joerg Wiedenmann, and he was kind enough to supply information about their lighting experiments. The high/low light trials were conducted using a 400-watt Aqua Medic Aqualine 13,000K metal halide lamp. The color experiments used two lamps. For the green and red filters, a 250-watt Osram Powerstar HQI-TS Daylight metal halide was employed. The blue filter was used in conjunction with a 250-watt BLV 20,000K Nepturion metal halide lamp. PAR intensity was standardized at 200 μmol·m^2·sec in these experiments. Thank you Dr. Wiedenmann for this additional data!

GREEN FLUORESCENT PIGMENT 512

- Host: *Acropora millepora*
- Pigment Clade: C2
- Pigment Type: Green Fluorescent Protein
- Fluorescence Best Induced by: Blue light (but see comments below)
- Light Intensity Required: Best fluorescence at 700 μmol·m^2·sec
- Photoconversion Possible: Yes (based on spectral characteristics of *Acropora tenuis*, below and observations of other pigments found in *Acropora millepora*).

Photoconversion is only seldom reported in scientific literature among corals containing Clade C pigments. Based on information offered by Papina et al. (2002), we could conclude that photoconversion does occur in Clade C2 pigments (found mostly in *Acropora* species). While Papina's data suggests photoconversion occurs in *Acropora tenuis*' Pigment 517, we see further evidence that it occurs in *Acropora millepora*, where exposure to Ultraviolet-A radiation enhances by two-fold the fluorescence of Pigment 513.

GREEN FLUORESCENT PIGMENT 514

- Host: *Montipora digitata*
- Pigment Clade: Unknown, but possibly C3
- Pigment Type: Green Fluorescent Protein
- Fluorescence Best Induced by: Blue light
- Light Intensity Required: Best fluorescence at 400 μmol·m^2·sec
- Photoconversion Possible: Unknown, but unlikely.

PHOTOCONVERSION

Photoconversion - the rearrangement of a chromophore by light energy resulting in shifts in perceived coloration - is known to occur in quite a number of green fluorescent pigments.

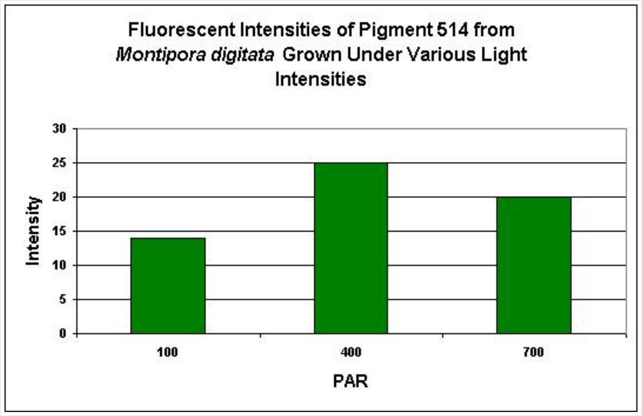

Figure 4. Effects of light intensity generated by a 13,000 K metal halide lamp. After D'Angelo et al., 2008.

Table 2. Photoconversion: Green Fluorescent Proteins convert to orange Kaede pigments (Clade D) upon exposure to ultraviolet/violet/blue light.

From	To	Host	Clade/Pigment	Activator
P-505	508/572	*Montastraea cavernosa*	D	? - But most likely blue light
P-505	506/566	*Ricordea florida*	D	? - But most likely blue light
P-508	575	*Dendronephthya* sp.	D	Blue Light @ 488nm
P-508	575	*Dendronephthya* sp.	D	UV-A at 366nm
~516	582	*Montastraea cavernosa*	D	Depth- light -related?
P-516	581	*Lobophyllia hemprichii*	D	UV @ 390nm/Violet Light ~400nm
P-517	574	*Ricordea florida*	D	UV/Violet Light
P-517	580	*Montastraea annularis*	D	UV/Violet Light
P-517	593	*Favia favus*	D	UV & Violet (350-420nm)
P-518	582	*Trachyphyllia geoffroyi*	D	UV - Violet Light (350-410nm)
P-519	580	*Montastraea cavernosa*	D	UV - Violet Light

PHOTOCONVERSION OF GFPS TO KAEDE PIGMENTS

Nature loves to confound us, and the conversion by light energy of one pigment to one of a different fluorescent emission certainly falls into this category. Photoconversion can occur in many pigments but is most well documented in those pigments classified as Kaede pigments. These are found mostly in suborder Faviina (which includes about 60 stony coral genera, including *Catalaphyllia*, *Favia*, *Lobophyllia*, *Montastraea*, *Mycedium*, *Trachyphyllia* and others), but also occurs in some soft corals (*Dendronephthya sp.*), and false corals(*Ricordea*).

The photoconversion from green to orange/red Kaede pigments requires blue light (see Table 2).

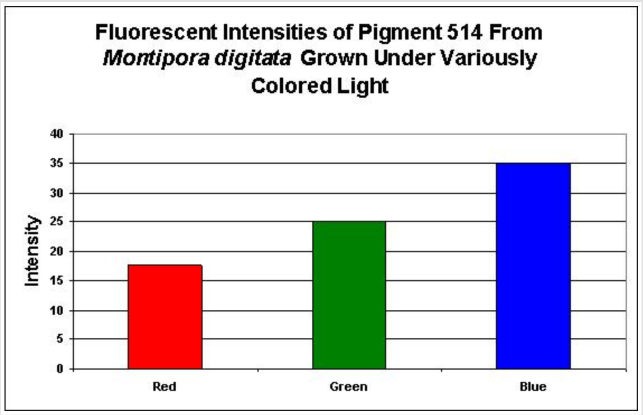

Figure 5. Effects of light color on generation of a green pigment of the popular aquarium color Montipora digitata.

Table 3. A case of light-induced pigment photobleaching in Clade D.

From	To	Host	Clade/ Pigment	Activator
P-518	None	"Pectiniidae"	D	~490nm to bleach

SPECTRAL SIGNATURES OF KAEDE PIGMENTS BEFORE AND AFTER PHOTOCONVERSION

Figures 7 through 9 demonstrate the spectral characteristics of Kaede pigments before and after photoconversion are induced by violet light.

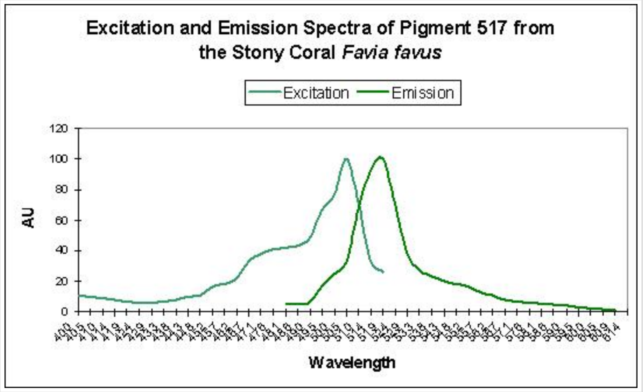

Figure 6. Favia favus contains a GFP that can be converted to an orange 'Kaede' pigment. This conversion is probably induced by 'strong' violet or blue light.

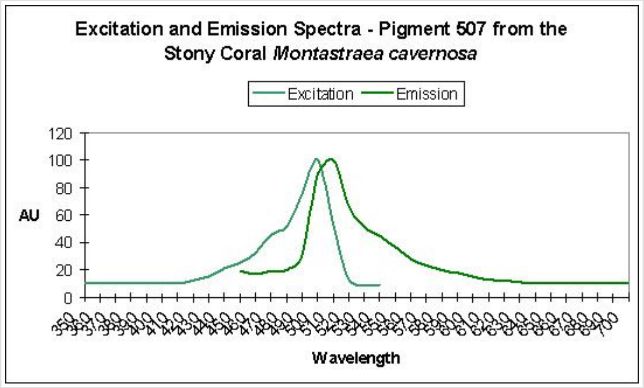

Figure 7. A green pigment showing little sign of photoconversion, but…

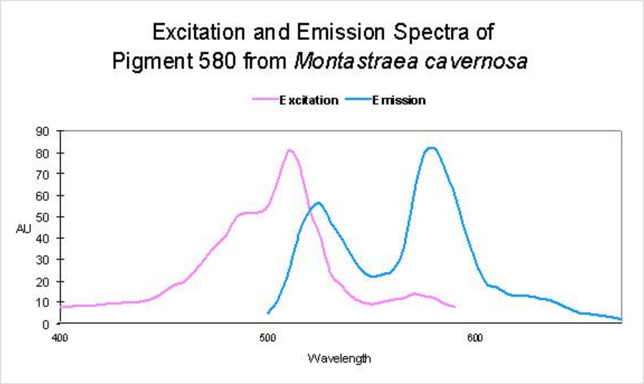

Figure 8. …the opposite is very apparent in this particular Montastraea specimen.

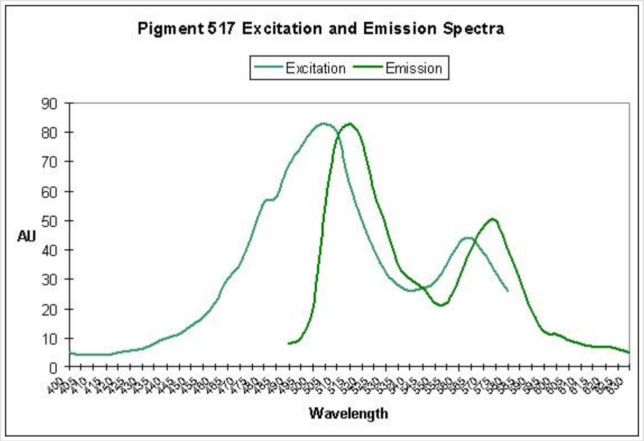

Figure 9. A case of photoconversion in progress in Ricordea florida. It is a Kaede pigment and photoconversion is likely mediated by violet/blue light. After Labas et al., 2002.

Table 4. In this instance, color conversion is due to a chemical process and can occur in darkness.

From	To	Host	Clade/ Pigment	Activator
P-520	611	*Entacmaea quadricolor*	A	Chemical Oxidation

PHOTOCONVERSION OF GFP-LIKE PIGMENTS TO DS-RED PIGMENTS

This type of pigment was originally isolated from the corallimorpharian *Discosoma*, but Ds-Red type pigments are found in anemones, false corals, zoanthids and stony corals.

Photoconversion is known to occur in some Ds-Red pigments, and is most well studied in *Discosoma* false coral species. Interestingly, the change in fluorescent from green (emission at 500 nm) to red (emission at 583 nm) does not require light energy and can occur in darkness - this change is mediated by chemical oxidation. However, a number of conversions from red to super red and super red to blue can occur when specimens are exposed to certain light wavelengths.

Table 4. In this instance, color conversion is due to a chemical process and can occur in darkness.

A POTENTIAL CASE OF PHOTOCONVERSION: A MINOR SHIFT OF GREEN FLUORESCENCE IN *ANEMONIA SCULATA*?

Figure 11 is offered in order to demonstrate a possible case of photoconversion in an anemone species (*Anemonia sculata*). The dual excitation and emission peaks suggest that the pigment is in, or has possibly completed, a shift of fluorescence of about 20 nm (from 522 nm to ~550 nm).

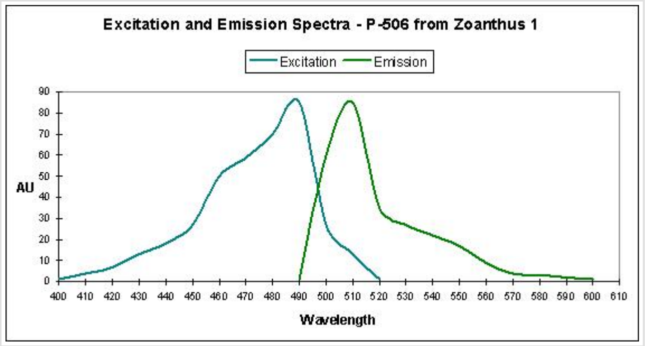

Figure 10. Laboratory experiments have resulted in this pigment changing its fluorescent emission from green to yellow when substituting only 3 amino acids in the protein. However, this has not been observed in the wild.

DISCUSSION

In both specific cases examined here, blue light induces the production of fluorescent proteins, while red light is the least effective in their production. However, we must note that light energy is not always the deciding factor in pigment production. For instance, some pigment production and/or conversion are due to chemical reactions and is independent of effects (or more properly, non-effects) of radiation - we see these cases in at least one instance involving an anemone and its Clade A pigment. Chemical oxidation may also play a part (but does not always) in color transformations see in some of the DsRed type pigments. However, where light energy is required, we should remember the light intensity categories described by D'Angelo:

- 'Very low' (80 µmol·m²·sec)
- 'low' (100 µmol·m²·sec)
- 'moderate' (400 µmol·m²·sec)
- 'high' (700 µmol·m²·sec)
- and a photoperiod of 12 hours.

The 'very low' and 'low' categories are quite easily achieved in home aquaria using modest means to produce light. The 'moderate' (400 µmol·m²·sec) category is found less commonly than the previous two categories. Producing this amount of light energy is quite possible when using some of the more powerful lighting sources such as metal halides, LEDs, and multiple fluorescent lamps. The highest category of 700 µmol·m²·sec is rarely seen, based on my observations. However, it is possible with selective placement of animals when using high output light sources. Bear in mind that photobleaching is possible in some pigments (for instance, the '*Dronpa*' pigment - see Table 3).

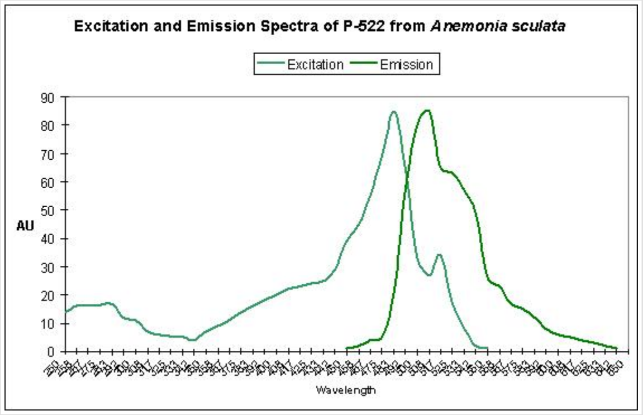

Figure 11. This pigment found in Anemonia sculata is of Clade A. Note the dual peaks and shoulders in the excitation and emission - this indicates possible photoconversion.

Lamps' spectral characteristics are important in promoting colorful pigments. In the two specific cases we examined this month, Pigment 512 (from *Acropora millepora*) and Pigment 516 (from *Montipora digitata*), blue light is by far the most efficient in making the coral animal produce green fluorescent proteins. But there are differences - those pigments of *Acropora millepora* (Clade C2) are not as likely to be promoted by other bandwidths (including green and red) than the *Montipora digitata* pigment (of Clade C3). It is unclear if these different responses to specific colors of light are common among their respective clades, and it will be interesting to see the results of future research. We know little about the responses of Clade C1 pigments (described as being isolated from *Meandrites* and *Eusmilia* specimens). Also, recall that the light intensity for the 'colored light' experiments was 200 µmol·m²·sec

Photoconversion of green pigments in Clade C2 pigments is known to occur (Clade C2 pigments are most often described as being present in *Acropora* and *Stylocoeniella* species). Ultraviolet radiation is known to cause a two-fold increase in the green fluorescence of Pigment 513 (but see comments about UV in the next paragraph).

Orange Kaede pigments are found mostly - not exclusively - in corals of the suborder Faviina (but are known to occur in at least one soft coral (*Dendronephthya*) and the false coral *Ricordea florida*). Conversion of green fluorescent proteins to the Kaede type orange pigments has been observed when the animal is exposed to ultraviolet-A radiation (UV-A), and violet/blue wavelengths. Care should be taken when experimenting with dosages of UV-A in attempts to promote coloration. Most plastic 'splash guards' are transparent to at least some UV-A wavelengths and it is usually not necessary to deliberately increase UV-A levels in order to induce production of pigments. With this said, there is some anecdotal information (based on my personal observations) that color of at least some pigments might increase in at least some coral species when UV radiation is *slightly* increased.

As I mentioned, the primary investigator responded with some useful information on the types of lamps used in the D'Angelo et al. experiments, and I have the appropriate lamps and filters on order. I'm most interested in observing the characteristics of the transmitted light and I'll report this information as it becomes available. Next time, we'll take a closer look at orange and red fluorescent proteins, and how they are affected by light intensity and spectra.

Comments? Questions? I am best reached via email at RiddleLabs@aol.com.

Table 1. A partial listing of Green Fluorescent Pigments (GFPs) with pertinent information.

Species	Pigment Name	Type	Suborder	Excit.	Emission	Clade
Acropora aculeus	aacuGFP1	GFP	Astrocoeiina	478	502	C2
Acropora aculeus	aacuGFP2	GFP	Astrocoeiina	502	513	C2
Acropora eurostoma	AeurGFP	GFP	Astrocoeiina	504	515	C2
Acropora millepora	amilGFP	GFP	Astrocoeiina	503	512	C2
Acropora millepora	amilFP512	GFP	Astrocoeiina	500	512	?
Acropora nobilis	anobGFP	GFP	Astrocoeiina	502	511	C2
Agaricia fragilis	afraGFP	GFP	Fungiina	494	503	D
Anemonia sculata	asFP499	GFP	Actinaria	?	499	A
Catalaphyllia jardineri	cjar	GFP	Faviina	509	517	D
Condylactis gigantea	cgigGFP	GFP	Actinaria	399/482	496	A
Dendronephthya sp.	dendGFP	GFP	Alcyonaria	494	508	D
Discosoma sp.	dis3GFP	GFP	Corallimorpharia	503	512	D
Echinophyllia echinata	eechGFP1	GFP	Faviina	497	510	D
Echinophyllia echinata	eechGFP2	GFP	Faviina	506	520	D
Echinophyllia echinata	eechGFP3	GFP	Faviina	512	524	D
Eusmilia fastigata	efasGFP	GFP	Meandriina	496	507	C1
Favites abdita	fabdGFP	GFP	Faviina	508	520	D
Galaxea fascicularis	gfasGFP	GFP	Meandriina	492	506	D
Galaxea fascicularis	Azami-Green	GFP	Meandriina	492	505	D
Heteractis crisperi	hcriGFP	GFP	Actinaria	405/481	500	A
Lobophyllia hemprichii	Eos	GFP	Faviina	506	516	D
Lobophyllia hemprichii	lhemOFP	GFP	Faviina	507	517	D
Meandrites meandrina	mmeanGFP	GFP	Faviina	487	515	C1
Montastraea annularis	monannGFP	GFP	Faviina	?	510	D
Montastraea cavernosa	mcavRFP	GFP	Faviina	504	517	D
Montastraea cavernosa	mcavGFP	GFP	Faviina	506	516	D
Montastraea cavernosa	P-510	GFP	Faviina	440	510	D
Montastraea cavernosa	mcav2	GFP	Faviina	505±3	515±3	D
Montastraea cavernosa	mcav3	GFP	Faviina	505±3	515±3	D
Montastraea cavernosa	mcav4	GFP	Faviina	505±3	515±3	D
Montastraea cavernosa	mcav6	GFP	Faviina	495	507	D
Montastraea cavernosa	r2	GFP	Faviina	?	522	D
Montastraea cavernosa	r3	GFP	Faviina	?	514	D

Species	Pigment Name	Type	Suborder	Excit.	Emission	Clade
Montastraea cavernosa	r4	GFP	Faviina	?	519	D
Montastraea cavernosa	r7	GFP	Faviina	?	505	D
Montastraea cavernosa	g1.2	GFP	Faviina	?	518	D
Montastraea cavernosa	g4	GFP	Faviina	?	518	D
Montastraea cavernosa	g6	GFP	Faviina	?	507	D
Montastraea faveolata	monfavGFP1	GFP	Faviina	440	510-520	D
Montipora digitata	mdigFP514	GFP	Astrocoeiina	508	514	?
Montipora efflorescens	meffGFP	GFP	Astrocoeiina	492	506	C3
Pectiniidae	Dronpa	GFP	Faviina	503	518	D
Platygyra lamellina	plamGFP	GFP	Faviina	502	514	D
Porites porites	pporGFP	GFP	Faviina	495	507	C3
Ricordea florida	P-510	GFP	Corallimorpharia	?	510	D
Ricordea florida	P-513	GFP	Corallimorpharia	?	513	D
Ricordea florida	P-517	GFP	Corallimorpharia	506	517	D
Ricordea florida	rfloGFP	GFP	Corallimorpharia	508	518	D
Ricordea florida	P-520	GFP	Corallimorpharia	?	520	D
Sarcophyton sp.	sarcGFP	GFP	Octocorallia	483	500	D
Scolymia cubensis	scubGFP1	GFP	Faviina	497	506	D
Scolymia cubensis	scubGFP2	GFP	Faviina	497	506	D
Stylocoeniella sp.	stylGFP	GFP	Astrocoeiina	485	500	C2
Trachyphyllia geoffroyi	P-518	GFP	Faviina	508	518	D

AQUARIUM CHEMISTRY

THE CARBONATE SYSTEM IN THE AQUARIUM, AND THE OCEAN, PART II: THE INTERACTING CARBONATE SYSTEM

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In this article we will discuss the major interactions of the carbonate system in sea water.

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Last month we discussed the various components of the carbonate system in sea water. We identified each parameter, mentioned typical values for these parameters in natural sea water, and discussed briefly why these parameters are important for aquarists to consider. This month we will begin to tackle how each of these components interacts with the others. Simply put, this article will examine how the carbonate system works.

THE CONTROL OF SEAWATER pH

The pH of typical surface sea water averages about 8.20 today, was nearer 8.30 during the preindustrial period, and was closer to 8.45 during the last glacial maximum (~20,000 years ago). These values are typical for oceanic pH over at least the last 23.5 million years. Indeed, mean oceanic pH has varied only within the range of about 8.2-8.45 during this period (Figure 1). One might ask why the pH of the shallow ocean has tended to stay within this range, even over comparatively long periods of time? Why isn't the pH of the ocean 6? Why not 10? The answer brings us to one of the most important interactions in the carbonate system.

Carbon dioxide gas is present in the atmosphere and dissolves into sea water according to its partial pressure, as described by Henry's Law:

$$[CO_2^*] = K_H \times pCO_2$$

where $[CO_2^*]$ is the concentration of dissolved CO_2 in the water, K_H is a Henry's Law constant for CO_2 in sea water (at a given temperature and pressure) and pCO_2 is the partial pressure of CO_2 in the atmosphere. The surface ocean will tend to take up a particular amount of CO_2 and that amount is directly proportional to the concentration of CO_2 in the air overlying that water. The exchange of CO_2 between the surface ocean and the atmosphere is constantly ongoing. That is, individual molecules of CO_2 are always moving from the surface ocean to the atmosphere and from the atmosphere to the surface ocean. These two pools exchange CO_2 back and forth all the time. If the ocean-atmosphere system is near equilibrium then the rate of exchange of CO_2 will be equivalent in both directions, ocean to atmosphere and atmosphere to ocean, ensuring no net change in the size of either pool.

This exchange can fall out of equilibrium if either the atmospheric or oceanic pool of dissolved CO_2 is altered. For example, if there is a net release of CO_2 into the atmosphere, increasing

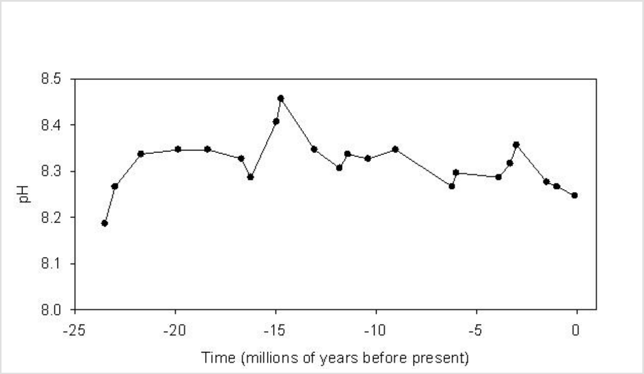

Figure 1. Mean surface ocean pH over the last 23.5 million years. After Pearson and Palmer, 2000.

the atmospheric concentration, the surface ocean will become undersaturated with respect to CO_2 given the new, higher atmospheric concentration. More CO_2 will dissolve from the atmosphere into the ocean than from the ocean to the atmosphere. The imbalanced exchange increases the amount of dissolved CO_2 in the ocean until the atmospheric and oceanic pools eventually reach a new equilibrium. In this case the amount of CO_2 dissolved in the surface ocean and in the atmosphere will both be higher at the new equilibrium than they were previously. Conversely, if there is a net removal of CO_2 from the atmosphere the reverse process will play out. The ocean will lose CO_2 to the atmosphere until the two reach a new equilibrium. In this case the amount of CO_2 dissolved in the surface ocean and in the atmosphere will both be lower than they were previously.

When $CO_2(g)$ dissolves into water it reacts with that water to produce carbonic acid (H_2CO_3). Adding an acid to sea water (or any solution), all else equal, reduces the pH. The release of CO_2 into the atmosphere results in an increase in the concentration of atmospheric CO_2, an increase in the amount of dissolved CO_2 in the surface ocean, an increase of the amount of carbonic acid, and a reduction of oceanic pH. The removal of atmospheric CO_2 results in a reduction in the concentration of atmospheric CO_2, a reduction of the amount of CO_2 dissolved in the surface ocean, a reduction of the amount of carbonic acid, and an increase in oceanic pH. In today's atmosphere the partial pressure of CO_2 is about 387 µatm and is very quickly increasing due to the burning of fossil fuels. We'll discuss how ocean chemistry is changing as a result of fossil fuel burning in a future article. In pure water the dissolution of CO_2 from today's atmosphere produces a mildly acidic solution with pH = 5.6 — much lower than the pH of sea water. Clearly there must be a component besides dissolved CO_2 that affects seawater pH.

Total alkalinity is that other component. While $CO_2(g)$ produces an acid when dissolved in water, certain minerals also dissolve into sea water and produce bases. The most important compounds that provide alkalinity in the ocean are the carbonates ($CaCO_3$, $MgCO_3$, etc.) and the silicates (Mg_2SiO_4, $KAlSi_3O_8$, etc.). When carbonates dissolve they release HCO_3^- and CO_3^{2-} ions into solution, providing alkalinity. Silicates tend to react with carbonic acid, derived from atmospheric CO_2, and likewise release HCO_3^- and CO_3^{2-} ions into solution. Borates also contribute a small portion of the total alkalinity (~2.9%) while other sources (e.g., phosphates) account for a very small portion (negligible for our purposes). Thus we have a dynamic system that controls seawater pH: $CO_2(g)$ in the atmosphere dissolves into sea water, produces an acid, and pushes the pH lower. Certain minerals dissolve in the ocean (or on land and are transported to the ocean in runoff), contribute to the total alkalinity, and push the pH higher. Sea water is buffered by opposing forces of dissolved CO_2 and total alkalinity (Figure 2). If the alkalinity in sea water were completely removed but atmospheric CO_2 remained unchanged the pH would fall to about 5.46 (at equilibrium with the atmosphere) due to the dissolution of CO_2. Alternatively, if the total CO_2 (TCO_2) were stripped from sea water but alkalinity remained unchanged the pH would rise to 10.6. It is the interplay between these two parameters that produces the pH we measure.

Over long time scales a variety of biological and geochemical processes work together to determine the amount of CO_2 dissolved in the atmosphere and the ocean as well as the total alkalinity of oceanic water. Feedbacks among these various mechanisms have kept *long-term* oceanic pH within about the range of 8.2-8.45 for at least the last 23.5 million years. Over short time scales, however, the pH of sea water can and does vary because of *short-term* variation in the amount of dissolved CO_2 or the total alkalinity.

The take-home message here is this: higher dissolved CO_2 yields lower pH whereas lower dissolved CO_2 yields higher pH, all else equal. pH and dissolved CO_2 are *inversely* related. Conversely, higher alkalinity yields higher pH while lower alkalinity yields lower pH, all else equal. pH and alkalinity are *directly* related.

More often than not the supplementation schemes that we use to control alkalinity in captivity (whether a buffer, two-part system, calcium reactor, etc.) affect both alkalinity and dissolved CO_2 at the same time. Hence the question of how a supplement or supplementation system affects tank pH becomes a more complex problem. We will consider these more complex interactions next month. For now let us consider just the basic relationship among dissolved CO_2, alkalinity, and pH mentioned above. Reducing dissolved CO_2 *or* increasing alkalinity raises pH. Increasing dissolved CO_2 *or* reducing alkalinity lowers pH.

The other parameters of the carbonate system, calcium and magnesium, do not directly affect seawater pH. Instead, it is the interaction between total CO_2 and total alkalinity that is responsible for determining the pH in our tanks, and in the ocean. In nature total alkalinity is nearly conservative in its behavior (i.e., varies simply due to altered salinity) over short timescales (< 100,000 yrs), thus it is primarily variation in dissolved CO_2 (and hence atmospheric CO_2) that determines oceanic pH. In our aquaria we have the opportunity to manipulate both dissolved CO_2 and alkalinity to arrive at a desired pH.

THE CONCEPT OF SATURATION STATE

Let's imagine a simple experiment. We fill a beaker with deionized water. Next we drop in a few pieces of solid calcium carbonate ($CaCO_3$). What will happen? As you may have guessed, over time some of the $CaCO_3$ will dissolve into the water. If we periodically measure the concentrations of dissolved calcium and carbonate, we'll see a pattern like that in Figure 3. Initially there will be no dissolved $CaCO_3$ in the water. When we add solid $CaCO_3$ it dissolves quickly at first. As the concentration of dissolved $CaCO_3$ increases over time the rate of net dissolution slows down. Eventually the concentration of dissolved $CaCO_3$ plateaus. Note that this does not mean that there is no longer any dissolution taking place, but that the rate of dissolution and precipitation are equal, producing no net change in concentration thereafter.

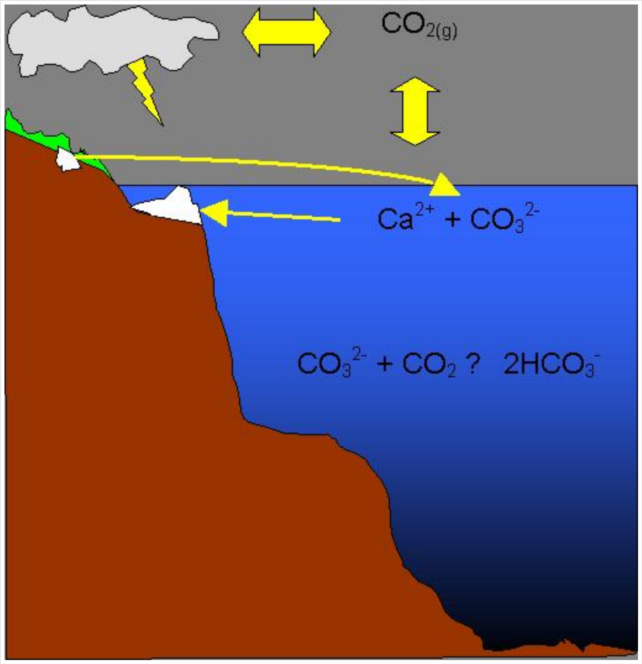

Figure 2. Carbon dioxide is exchanged between the atmosphere and the shallow ocean. Carbonates and other minerals dissolve into sea water, or are transported there in runoff, providing alkalinity. The interaction between dissolved CO_2 and alkalinity largely determine seawater pH at any given moment.

Let's now consider a similar experiment. To begin this experiment, instead of putting in solid $CaCO_3$ we add a large amount of the dissolved components (e.g., a calcium chloride salt and a sodium carbonate salt). What will happen this time? This time we'll see the reverse process: the precipitation of solid $CaCO_3$ from the dissolved components. As seen in Figure 4, the concentrations begin at some high level and the rate of precipitation is fast. Over time the concentrations drop as does the rate of net precipitation. Eventually the concentration of dissolved $CaCO_3$ plateaus, indicating that the rate of precipitation is equal to the rate of dissolution.

If we plot the data for both of these experiments on the same graph, we see that they converge to the same value for dissolved $CaCO_3$ (Figure 5).

The value that these two curves converge to is the solubility of $CaCO_3$ in deionized water, at a given temperature and pressure. We would see a similar pattern were we to conduct these experiments with any mineral. The parameters that would change for a different mineral would include the value that the curves converge to (= the solubility) and the amount of time required to reach that value. We can describe the solubility of $CaCO_3$ using the following equation,

$$K_{sp} = (Ca^{2+})(CO_3^{2-})$$

where (Ca^{2+}) and (CO_3^{2-}) are the activities of calcium and carbonate ions, respectively, and K_{sp} is the solubility product of $CaCO_3$. As we mentioned last month in our discussion of pH, ion activity is equivalent to ion concentration in solutions with very low ionic strength (= very little dissolved minerals). In a salty solution like sea water the activity of Ca^{2+} and CO_3^{2-} will be much lower than the concentrations because of interactions

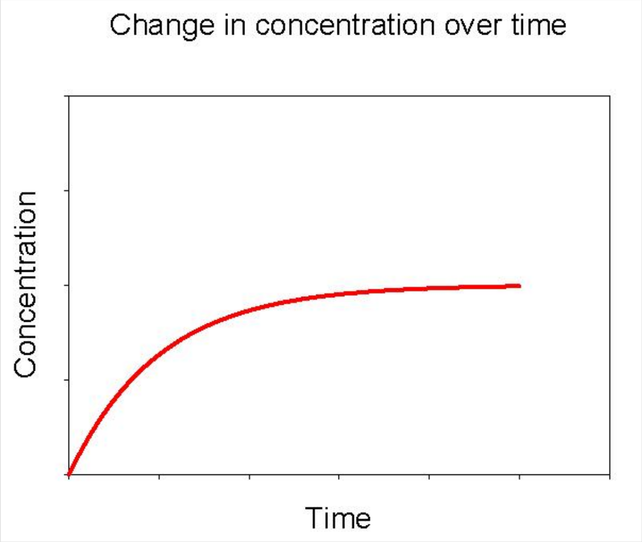

Figure 3. Change in concentration of dissolved $CaCO_3$ over time upon adding solid $CaCO_3$ to a solution initially devoid of dissolved $CaCO_3$.

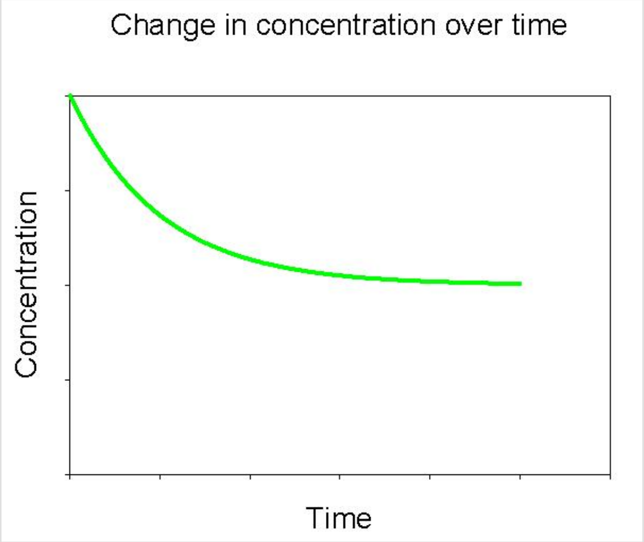

Figure 4. Change in concentration of dissolved $CaCO_3$ over time after initially adding a large amount of dissolved $CaCO_3$ to solution.

with other ions. For convenience, the solubility product (K_{sp}) can be transformed for use in sea water. Calcium and carbonate ion activities are replaced with concentrations yielding an *apparent* solubility product (K_{sp}`):

$K_{sp}` = [Ca^{2+}][CO_3^{2-}]$

K_{sp}` represents the value to which the curves above converged. At a given temperature and pressure it is simply a constant, a number that represents how much $CaCO_3$ we would expect to dissolve. In the first experiment above, our solution was initially undersaturated with $CaCO_3$ and took time to come up to saturation as $CaCO_3$ dissolved. In the second experiment our solution was initially supersaturated and took time to come down to saturation as $CaCO_3$ precipitated. We can express the degree of saturation (supersaturation, undersaturation, or saturation) by taking a ratio of the amount of $CaCO_3$ actually dissolved in a water sample to the value that we would expect to be dissolved at equilibrium. This ratio is called the saturation state, and is given the symbol Ω:

$\Omega = [Ca^{2+}][CO_3^{2-}]/K_{sp}`$

If $\Omega = 1$ then our solution is exactly at saturation—there is the same amount of $CaCO_3$ dissolved as we would expect to dissolve. We'd be at the point where the two curves above converge. If $\Omega < 1$ then the solution is understurated and we would expect net dissolution, like in the first experiment. If $\Omega > 1$ then the solution is supersaturated and we would expect net precipitation, like in the second experiment. The saturation state simply compares the amount of $CaCO_3$ dissolved in the water to the amount that we expect should dissolve.

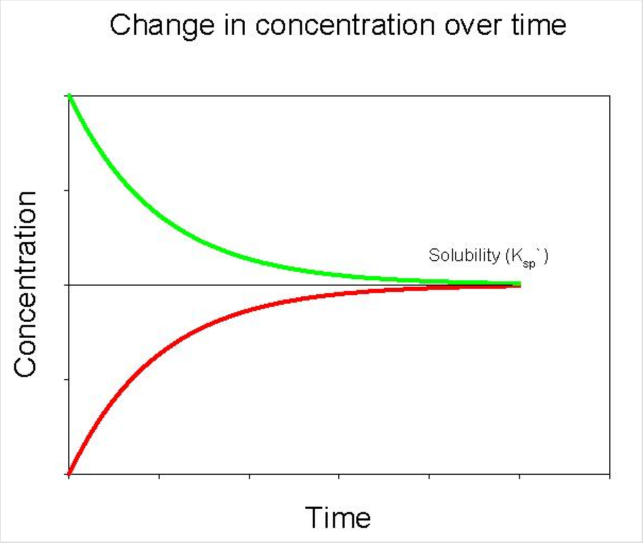

Figure 5. Change in concentration of dissolved $CaCO_3$ over time upon adding solid $CaCO_3$ to a solution initially devoid of dissolved $CaCO_3$ (red) and after initially adding a large amount of dissolved $CaCO_3$ to solution (green).

Rates of $CaCO_3$ dissolution and precipitation are proportional to the saturation state (Mucci, 1983). That is, $CaCO_3$ dissolves faster if the saturation state is really low than if the saturation state is just a little low, like in our first experiment above. Likewise, $CaCO_3$ precipitates faster if the saturation state is really high instead of just a little high, like in our second experiment above.

Saturation state will change if any of the three parameters used to calculate it change:

1. K_{sp}`
2. Ca^{2+} concentration
3. CO_3^{2-} concentration.

K_{sp}`, representing the amount of $CaCO_3$ we expect to dissolve, can be altered by changes in temperature or pressure. $CaCO_3$ is more soluble at low temperatures and less soluble at high temperatures, unlike most solids. $CaCO_3$ is also more soluble at very high pressure than at lower pressure. While the temperature effect can be important in aquaria in areas of local high temperature (e.g., on heaters, inside pumps) or over wide geographic areas in nature (e.g., tropics to polar regions), the day-to-day or seasonal variation in temperature typical on a coral reef or in an aquarium has only a modest impact on K_{sp}`. The effects of pressure are important in considering $CaCO_3$ solubility in the shallow ocean compared to the deep sea, but not on coral reefs or in aquaria.

Calcium concentration is conservative in sea water (as discussed in Part I). Oceanic calcium concentration has in fact changed quite significantly over long timescales (millions of years), but over geologically short time scales (< 100,000 yrs) Ca^{2+} concentration does not and truly cannot change too much in the ocean. This is largely a result of the comparatively high background concentration of Ca^{2+} in the ocean. To significantly change the Ca^{2+} concentration of sea water requires the precipitation or dissolution of truly massive quantities of minerals. Even at maximal rates of dissolution or precipitation in nature, typically millions of years are required to substantially change the calcium concentration of sea water. Calcium concentration can, however, be manipulated over a wide range in aquaria.

The last parameter, CO_3^{2-} concentration, is the one that is most likely to cause significant variation in Ω both in nature and in aquaria. Because of the nearly conservative behavior of alkalinity in the ocean, for a given salinity CO_3^{2-} concentration varies primarily as a function of pH, and hence of dissolved CO_2. In captivity we can manipulate both alkalinity and pH (via CO_2), yielding a potentially very large range of values for CO_3^{2-} concentration in aquaria. An increase in either alkalinity or pH will result in an increase in CO_3^{2-} concentration. Conversely, a decrease in either alkalinity or pH will result in a decrease in CO_3^{2-} concentration (Figure 6).

By manipulating Ca^{2+} concentration, total alkalinity, and pH we are able to arrive at a wide range of values for Ω in aquaria, but what sort of range? The answer depends heavily on the type of $CaCO_3$ mineral under consideration.

CALCIUM CARBONATE MINERALOGY

The compound $CaCO_3$ can form several different minerals. These different forms (referred to as polymorphs) have different physical arrangements of ions in the lattice structure of the crystals. While it may at first seem strange that the same ions can be used to form different minerals, elemental carbon (C) provides an example that may be more familiar. Pure carbon can assemble into several different crystalline structures. Among these different forms are graphite (i.e., what's in pencils) and diamond. The spatial arrangement of carbon atoms in the crystal lattice of these two minerals is distinct. In graphite the carbon atoms make a series of sheets, like the pages of a book. It's easy to separate those sheets, hence why graphite readily leaves smudges when rubbed against a surface. Diamond, unlike the sheets of carbon atoms in graphite, is a cubic crystal which gives diamond its strength. The different physical arrangement of carbon atoms gives graphite and diamond very different physical properties. Graphite is very soft while diamond is the hardest known mineral.

Similarly, $CaCO_3$ can form crystals that have different arrangements of the two ions. The distinct arrangements give the minerals somewhat different physical properties. The three most common polymorphs of $CaCO_3$ produced by calcifying organisms are low-magnesium calcite (low-Mg calcite), high-magnesium calcite (hi-Mg calcite) and aragonite. Both hi- and low-Mg calcite have a rhombohedral lattice structure, but Mg^{2+} replaces many of the Ca^{2+} in the lattice of hi-Mg calcite. Aragonite has an orthorhombic lattice. Other polymorphs such as vaterite occur in some organisms, but are relatively rare in the completed shells/skeletons.

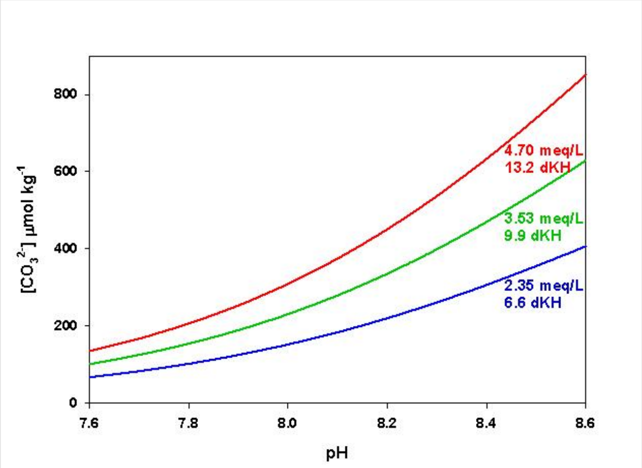

Figure 6. Effects of pH on CO_3^{2-} concentration at three levels of alkalinity. Calculated using co2sys (Lewis and Wallace, 1998) assuming S = 35, T = 25 °C, P = 1 atm, and negligible total phosphate and silicate concentrations.

One of the most important physical properties that changes among these polymorphs is the solubility. We defined the saturation state, Ω, as the ratio of the amount of $CaCO_3$ dissolved in a water sample to the amount we expect to dissolve. Since the various $CaCO_3$ polymorphs have different solubilities, we must us the K_{sp}` that is appropriate for the mineral we are interested in. Low-Mg calcite and aragonite are reasonably similar in their composition whether they are made by different organisms or precipitated abiotically. Therefore, their solubility has been well characterized in sea water over a range of conditions. Hi-Mg calcite is truly a mixed solid made of mostly $CaCO_3$, but also a significant quantity of $MgCO_3$. Calcite that is < 4 mol % $MgCO_3$ is typically classified as low-Mg calcite whereas calcite that is > 4 mol % $MgCO_3$ is classified as hi-Mg calcite. Usually hi-Mg calcite formed by marine organisms ranges from about 8-20 mol % $MgCO_3$ while low-Mg calcite is not more than a few mol % $MgCO_3$. Magnesium carbonate is much more soluble than $CaCO_3$ in sea water. Hence, the more Mg^{2+} there is in the hi-Mg calcite, the more soluble it will be. The proportion of Mg^{2+} in hi-Mg calcite can be variable depending on the conditions in which it was formed and/or the organism that formed it, leading to differences in solubility. As a result, the K_{sp}` for hi-Mg calcite is not as well characterized as for low-Mg calcite or aragonite. Roughly speaking, however, hi-Mg calcite is about 1.5-1.7 times as soluble as aragonite in modern sea water while aragonite is about 1.5 times as soluble as low-Mg calcite (Millero, 2006).

Coccolithophores, a group of phytoplankton, produce low-Mg calcite as do some molluscs, some crustaceans, some bryozoans and foraminiferans. Coccolithophores are especially important to global patterns of calcification and primary production (much of the O_2 I am breathing as I write this and that you are breathing while you read this came from coccolithophores). The Cretaceous period at the end of the Mesozoic is named for the massive limestone deposits created by the ancestors of today's coccolithophores (the root *creta* is Latin for chalk). Of the organisms we are likely to keep in aquaria, some crustaceans (e.g, crabs, shrimps) may produce low-Mg calcite as do foraminiferans. Stony corals and tridacnid clams are perhaps the most important producers of aragonite to aquarists. Many molluscs (e.g., snails, bivalves, pteropods) produce aragonite as do the codiacean algae *Halimeda*, *Penicillus* (Mermaid's Brush), and *Udotea*. Most other calcifying organisms, including coralline algae, some molluscs, some crustaceans, soft corals, and echinoderms produce hi-Mg calcite (Table 1). While these are the principal $CaCO_3$ polymorphs produced by each group, many organisms can produce at least small amounts of other polymorphs. Some, such as some molluscs, may produce both aragonite and calcite in different parts of the shell or during different life stages (e.g., American oysters, *Crassostera virginica*, produce aragonitic shells as larvae and primarily calcitic shells as adults).

Certain variations in seawater chemistry can strongly influence which polymorph of $CaCO_3$ some organisms produce, but have much less influence on others. For instance, coralline algae, which produce hi-Mg calcite in today's hi-Mg sea water, can be induced to produce low-Mg calcite by growing them in low-Mg sea water. Scleractinian corals can also be induced to produce some low-Mg calcite in low-Mg sea water, but they continue to produce principally aragonite in such a scenario. Why most organisms produce a particular polymorph of $CaCO_3$ instead of another (e.g., why do coralline algae produce principally calcite and not aragonite; why do corals produce principally aragonite and not calcite?) is poorly understood at present, but is an intriguing research question.

As we can see, most of the organisms we are interested in keeping in aquaria produce either aragonite, hi-Mg calcite, or both. Few are likely to produce low-Mg calcite. Since the solubility of aragonite is well characterized over a range of temperatures, pressures, and salinities but that of hi-Mg calcite is not, the K_{sp}\` for aragonite is the most useful to us. As mentioned above, hi-Mg calcite (from reef organisms) is roughly 1.5-1.7 times as soluble as aragonite. Hence, we would expect hi-Mg calcite to begin dissolving when the aragonite saturation state (Ω_{arag}) falls below *roughly* 1.5-1.7. We would not expect net aragonite dissolution until Ω_{arag} falls below 1.0.

In today's ocean Ω_{arag} around most coral reefs falls within the range of about 3-4, on average. In other words, there is about 3-4 times as much $CaCO_3$ dissolved in the water as we would expect based on the solubility of aragonite. Notice that this range is also well above the threshold of roughly 1.5-1.7 where we expect to start seeing dissolution of hi-Mg calcite. Sea water in the shallow ocean near corals reefs is, on average, supersaturated with respect to both aragonite and hi-Mg calcite, favoring the precipitation of $CaCO_3$.

As we mentioned last month in our discussion of pH, however, it is not unusual to see a fair amount of pH variation on coral reefs over the course of 24 hrs. During the daytime photosynthesis removes CO_2, raising pH in the water overlying the reef. The increase in pH shifts the inorganic carbon species toward CO_3^{2-}, increasing Ω_{arag} (Figure 7). Daytime pH may rise to 8.3-8.6, resulting in Ω_{arag} as high as 5-7. At night the production of CO_2 from respiration can reduce reef pH as low as 8.1-7.8, causing Ω_{arag} to fall as low as 1.5-2.5 (Figure 7). Notice that these nighttime values for Ω_{arag} are near or even slightly below those where we expect to begin to see dissolution of hi-Mg calcite. Dissolution of carbonates can and does happen on coral reefs today, especially at the lower pH values experienced at night.

Depending on how we manipulate Ca^{2+} concentration, alkalinity, and especially pH in our aquaria we can obtain values for Ω_{arag} significantly above as well as below the range typical on a coral reef. We can produce conditions that favor the rapid precipitation of $CaCO_3$ (significant supersaturation), and we can produce conditions that favor dissolution (undersaturation).

Given that there's so much "extra" $CaCO_3$ dissolved in sea water, we might expect that a lot of it should spontaneously precipitate out, just like in the experiment we discussed above. Spontaneous precipitation might be expected based on the thermodynamic solubility, but by and large it doesn't happen, at least not quickly. Well, we might ask, why in the world not?

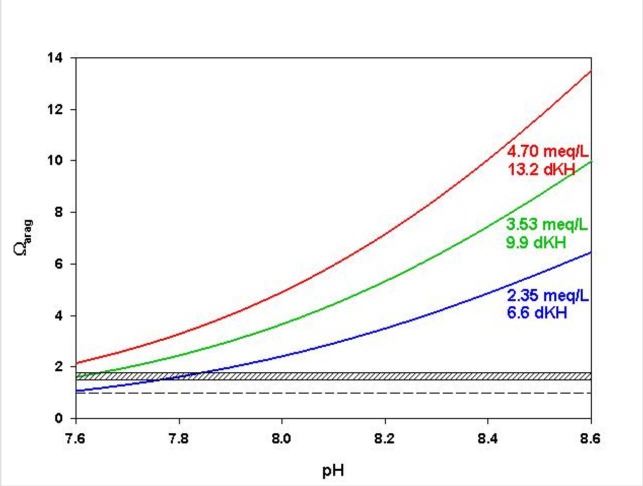

Figure 7. Effects of pH on Ωarag at three levels of alkalinity. Dashed line indicates threshold for aragonite dissolution. Hashed box indicates approximate threshold for hi-Mg calcite dissolution. Calculated using co2sys (Lewis and Wallace, 1998) assuming S = 35, T = 25 °C, P = 1 atm, and negligible total phosphate and silicate concentrations.

Table 1. Principal calcium carbonate polymorph for various coral reef associated taxa.

Taxa	**Principal $CaCO_3$ Polymoph**
Scleractinian corals	Aragonite
Tridacnid clams	Aragonite
Halimeda, *Penicillus*, *Udotea*	Aragonite
Gastropods	Aragonite or hi-Mg calcite
Coralline algae	Hi-Mg calcite
Echinoderms	Hi-Mg calcite
Soft corals	Hi-Mg calcite
Crustaceans	Hi- or low-Mg calcite
Foraminiferans	Low-Mg calcite

CALCIUM CARBONATE PRECIPITATION

Heat up a beaker of water and dissolve a large amount of table salt (NaCl) in it. Let the beaker cool down and you will have a supersaturated solution. If you then toss in a seed crystal, or anything else that salt crystals can grow on (a nucleation site) NaCl crystals will spontaneously precipitate out of solution. Scoop up a beaker of sea water next to a coral reef (supersaturated with respect to all three major polymorphs of $CaCO_3$), toss in a seed crystal, seal it up, and put it on a shelf. You could wait months or even years all the while seeing very little happen. There must be something getting in the way of $CaCO_3$ precipitation in sea water despite the fact that precipitation is thermodynamically favored.

One of the major obstacles to $CaCO_3$ precipitation in today's sea water is the high Mg^{2+} concentration of that water. Mg^{2+} exerts two distinct effects on $CaCO_3$ precipitation. The first effect is an ion-pairing effect. Magnesium forms ion pairs with CO_3^{2-} ions in solution. These ion pairs are short-lived, constantly forming and breaking apart, but they do affect the activity of CO_3^{2-} in solution. An increase in Mg^{2+} concentration means that a larger proportion of the CO_3^{2-} will occur as $MgCO_3^0$ pairs, decreasing the activity of CO_3^{2-}. In normal sea water, ~55% of the CO_3^{2-} exists as ion pairs with Mg^{2+}. Another ~10% pairs with Na^+ ions and ~10% with Ca^{2+} leaving ~25% of the total as the free ion. By forming ion pairs with CO_3^{2-}, Mg^{2+} does not make the precipitation of $CaCO_3$ impossible, but it does slow it down significantly. Interestingly, by lowering the activity of CO_3^{2-} in solution, Mg^{2+} allows more $CaCO_3$ to dissolve than otherwise would. In this way, Mg^{2+} ions allow a large amount of $CaCO_3$ to dissolve into sea water if that sea water becomes undersaturated with respect to aragonite and/or calcite, such as happens in the deep sea. Those Mg^{2+} ions also significantly slow down the rate of $CaCO_3$ precipitation from sea water when that sea water becomes supersaturated, like in the shallow ocean. The ion-pairing between Mg^{2+} and CO_3^{2-} slows down the rate of $CaCO_3$ precipitation, but it alone is insufficient to explain why we don't see more abiotic $CaCO_3$ precipitation in sea water.

The second and more important effect that magnesium ions exert on CaCO3 precipitation is that they poison the surface of growing crystals. Magnesium ions are attracted to the carbonate ions in $CaCO_3$ surfaces, much like calcium ions are. As they attach to these surfaces they can become incorporated into the growing crystal. Magnesium ions, while chemically similar to calcium ions (Mg is in the same period and directly above Ca on the periodic table), are the smaller of the two. Think of baseballs vs. softballs. While magnesium ions fit into the crystal lattice of $CaCO_3$ well enough to be incorporated, they disrupt the pattern. Imagine building a brick wall: if all the bricks are the same size, it's easy to construct the wall. Now imagine that every now and again a smaller brick is randomly inserted in place of a normal sized one, but the same amount of mortar is used to hold the bricks together. The wall would end up very misshapen in short order, and eventually would deviate so far from the original pattern it would be difficult to continue building at all. Magnesium ions disrupt the precipitation of $CaCO_3$ in much the same way. They turn the growing surface of $CaCO_3$ crystals into odd shapes where it is not easy for $CaCO_3$ to precipitate. The higher solubility of $MgCO_3$ as compared to $CaCO_3$ also means that such crystals will more easily redissolve, inhibiting $CaCO_3$ precipitation even if it is thermodynamically favored.

While magnesium ions disrupt the lattice structure of calcite, they fit well enough that they can be incorporated as a fairly large proportion of the crystal. Hi-Mg calcite is structurally the same as low-Mg calcite, except that many of the calcium ions have been replaced by magnesium ions, yielding to irregularities in the lattice structure. The ions in the aragonite lattice, however, are somewhat more widely spaced than in the calcite lattice. They are spaced widely enough that Mg^{2+} only rarely coprecipitates with Ca^{2+} in aragonite crystals. While some aragonite skeletons/shells contain more Mg^{2+} than others, none approach the magnesium content of hi-Mg calcite crystals. When the molar Mg/Ca ratio of a solution is < 1 low-Mg calcite tends to form preferentially. If the Mg/Ca ratio is increased then the calcite will incorporate progressively more and more Mg2+ into the crystals. At a Mg/Ca ratio between 1 and 2 we see the formation of primarily hi-Mg calcite. When the Mg/Ca ratio is increased above 2 we begin to see aragonite precipitation, along with hi-Mg calcite. As the Mg/Ca ratio is increased further still to 3, 4, 5 (or the 5.2 in today's ocean) aragonite constitutes a larger proportion of the $CaCO_3$ precipitated while the Mg content of the hi-Mg calcite continues to climb. If we raise the Mg^{2+} concentration high enough we can essentially inhibit calcite precipitation (as hi-Mg calcite) entirely. Aragonite precipitation, however, will continue to occur even at high Mg^{2+} concentration. Magnesium ions directly inhibit calcite precipitation by poisoning the growing crystal. Magnesium ions do not significantly affect aragonite precipitation, though low magnesium concentration favors the precipitation of calcite, outcompeting aragonite crystals for calcium and carbonate ions.

By maintaining the Mg^{2+} concentration in our aquaria near that in today's sea water we can favor the precipitation of aragonite and hi-Mg calcite, the polymorphs of $CaCO_3$ produced by nearly all of the organisms we keep in captivity. We can also minimize the rate of abiotic $CaCO_3$ precipitation on pumps, heaters, and elsewhere in our aquaria. Sufficiently high Mg^{2+} concentration helps to maintain significant $CaCO_3$ supersaturation in our aquaria, and the shallow ocean, and encourages rapid calcification and growth in the vast majority of calcifying organisms we might keep. Organisms that normally produce low-Mg calcite in today's ocean either live in polar regions (low-Mg calcite is the favored polymorph at a temperatures < 2 °C) or do so in spite of the high Mg^{2+} concentration. That some organisms produce a thermodynamically unfavorable mineral for their shells/skeletons reminds us that biologically mediated calcification is fundamentally dependent upon the physiological capacities of the organisms performing the calcification, though environmental factors influence those capacities.

CONCLUSION

In this article we discussed the major interactions of the carbonate system in sea water. We began by exploring why sea water has the pH that it does. Seawater pH at any given moment is determined almost entirely by the interaction between total alkalinity and total CO_2/dissolved CO_2. The other parameters of the carbonate system, calcium and magnesium, do not directly affect pH. In order to appreciate why $CaCO_3$ precipitates from sea water at all, we went next to the concept of saturation state, Ω. The polymorph of $CaCO_3$ is a critical consideration in defining Ω though, so our discussion turned to $CaCO_3$ mineralogy next. The organisms we keep in captivity generally produce aragonite or hi-Mg calcite, so the aragonite saturation state (Ω_{arag}) is the one most useful to us. Calcium concentration and alkalinity tend not to vary much in nature at a given salinity, so variation in Ω_{arag} is caused principally by the effects of varied pH on CO_3^{2-} concentration. In captivity we can provide a much wider range of values for Ω_{arag} because we are able to manipulate each of calcium concentration, alkalinity and pH. We can provide conditions that favor rapid $CaCO_3$ precipitation, and conditions that favor dissolution. Lastly we saw that Mg^{2+} plays a critical role in $CaCO_3$ dissolution and precipitation. The high concentration of Mg^{2+} in today's ocean allows a large amount of $CaCO_3$ to dissolve if sea water becomes undersaturated with $CaCO_3$, but inhibits $CaCO_3$ precipitation if sea water then becomes supersaturated. Magnesium ions help to keep $CaCO_3$ supersaturated in our aquaria and in the shallow ocean by inhibiting abiotic precipitation of $CaCO_3$. The high Mg^{2+} concentration in today's sea water also favors the precipitation of aragonite and hi-Mg calcite over low-Mg calcite, helping to favor the calcification and growth of the majority of the calcifying organisms we might keep in captivity. Next month we'll discuss methods available to manipulate these parameters in aquaria and explore how different types of manipulations affect the entirety of the carbonate system.

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FEATURE ARTICLE

AN OVERVIEW OF SYMBIOTIC RELATIONSHIPS OF THE CARIBBEAN, WITH EMPHASIS ON SHRIMPS PERICLIMENES YUCATANICUS [IVES, 1891] AND PERICLIMENES PEDERSONI [CHACE, 1958] (DECAPODA: CARIDEA: PALAEMONIDAE), AND HOST ANEMONES

By Michael Maddox

Researching these relationships helps to illustrate how important life is to other life, and what an incredibly complex ecosystem Earth supports, and that ecosystem should not be taken for granted.

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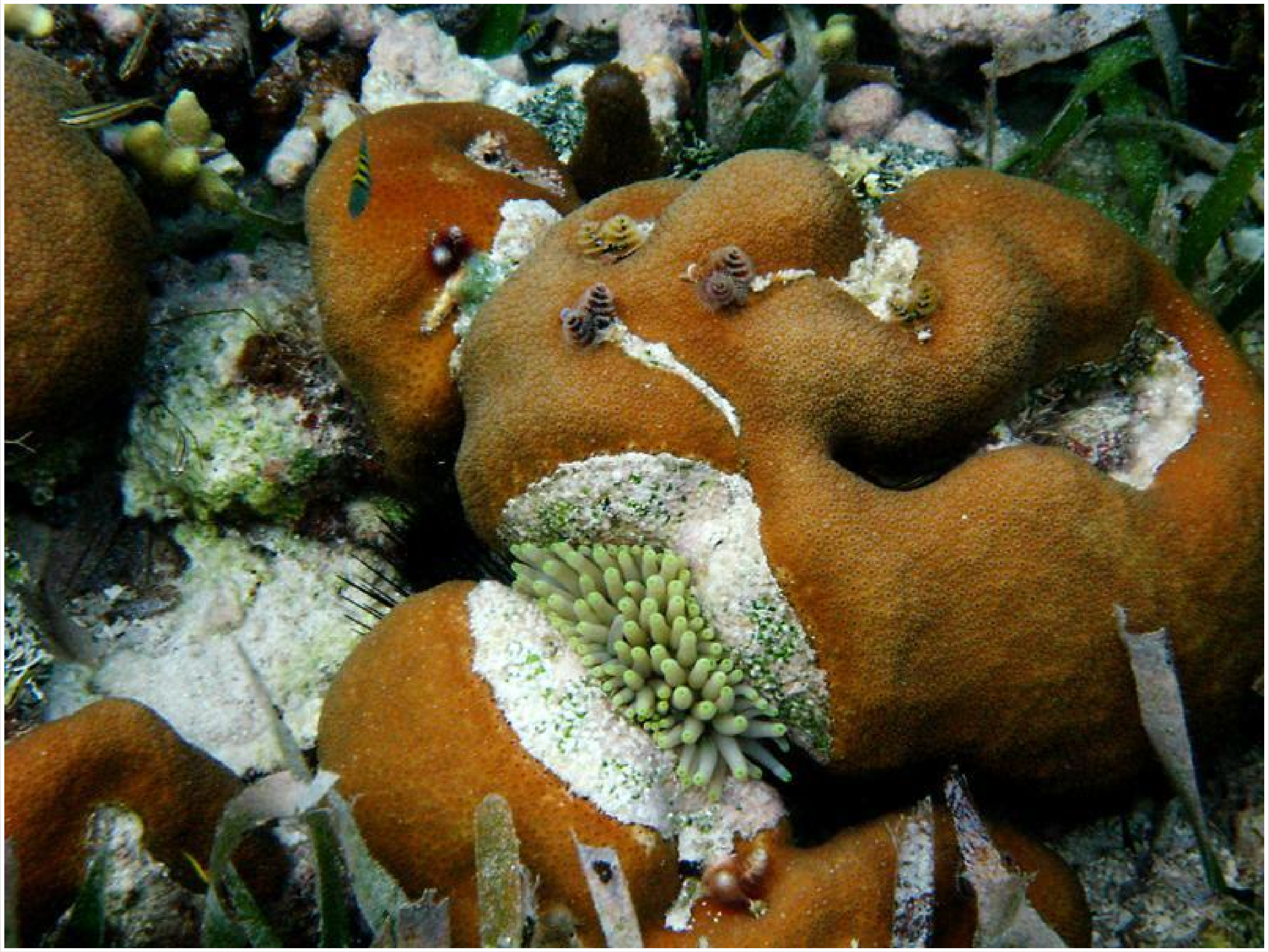
Symbiotic relationships are an icon of marine aquarium keeping, and no wonder – who hasn't paused when presented with clownfish happily swimming among the tentacles of their host anemone? Symbiotic relationships are found worldwide, in every habitat, and can be described as two different organisms living together in one of four ways:[1] Commensalism is a relationship of an animal, plant, fungus, etc. living with, on, or in another, without injury to either. Mutualism is a relationship between two species of organisms in which both benefit from the association. Parasitism is a relation between organisms in which one lives as a parasite on another. Lastly, phoresis is a relationship in which a host animal provides transportation for the symbiont.[2] There are numerous symbiotic relationships to be found in the Caribbean, and a portion of these relationships will be discussed here, with emphasis placed on relationships I personally observed in the Drowned Cayes, Belize, Central America.

The commensal relationship between shrimp Pederson's anemone shrimp (*Periclimenes pedersoni*) and the spotted or Yucatan anemone shrimp (*Periclimenes yucatanicus*) is easily observed in the wild, but seldomly (though becoming more common) in the home aquarium. I was able to personally observe both species residing commensally in host anemones in their native habitats, and would like to share information about their natural life with the marine aquarists that are so inclined. The shrimp belong to the family Palaemonidae, and are easily distinguished by their long, serrated rostrums, and small average size (to ~3.5 cm, depending on the species). *Periclimenes* species are found in tropical waters of the Caribbean and the Western Atlantic. (Spotte & Bubucis, 1997) Shrimp from this genus are known for their 'cleaning' behavior, removing and consuming parasites from fish, though I was unable to personally observe or record this behavior. (Calado, et al. 2003) Shrimp of this family are virtually always associated commensally with coral, anemones, and occasionally corallimorpharians (Williams & Williams, 1981). Coloration may vary even between members of the same species, as it can be changed to camouflage with the shrimp's host, making members of this family sometimes difficult to identify visually. (Fenner, 2000-2007) *Periclimenes* species usually prefer a few select species of sea anemone to inhabit commensally. These species include the bulb-tipped anemone (*Entacmaea quadricolor*), The condylactys anemone (*Condylactys gigantae*), *Bartholomea annulata, Aiptasia pallida,* and *Stichodactyla helianthus.* The bulb-tipped and condylactys are hardy species of anemone that are commonly kept by the home aquarist. All commensal shrimp benefit their hosts by providing a nitrogen source, preventing nitrogenous limitation of the zooxanthellae in the host's tissues. (Spotte, 1995)



A window into nature that I feel illustrates how life depends on life. These species would not linger or could not live at this location if not for the scleractinian coral specie(s) in the photograph.

Anemones, in turn, provide a refuge for the shrimp, as well as a potential food source both from the host's tentacles, and from any prey killed by the host. (Fautin, et all. 1995) Although both species potentially benefit one another, the anemone benefits much less than the shrimp, and due to the transient nature of the shrimp, may not benefit at all. Thus, the symbiosis is defined as a commensal relationship. (Spotte, 1995, Fautin, et al. 1995)

Pederson's anemone shrimp is found off the Caribbean coast, always living commensally with a host *Condylactys* or *Bartholomea* spp. anemone, and (rarely) with *Euphyllia* corals (another common aquarium species). However, the shrimp does not necessarily stay with one host throughout its life; instead it appears to change hosts frequently. (Chace, 1958, Manken, 1972) Very little information is available regarding this species.

The Yucatan anemone shrimp has very distinctive pink abdominal markings, and is most often seen residing in *Condylactys gigantae* in the Caribbean. However, it has other host anemones: *B. annulata, A. pallida,* and *Lebrunia dannae.* (Williams & Williams, 1981, Spotte, 1997) *P. yucatanicus* is unusual in the fact that it is also known to live commensally in the corallimorpharian anemone *Rhodactis sactithomae. P. yucatanicus* is the only Periclimenes species know to associate with *R. sactithomae*. It is unlikely a corallimorpharian is able to provide the same level of protection as it lacks the nematocysts of the other anemone species, so this finding is not understood. *P. yucatanicus* can be sexed fairly easily when seen in pairs (a common occurrence) as the female is, on average, 5mm larger than the male. (Spotte, 1997) All members of *Periclimenes* are known as 'cleaner shrimp', and have a mutualistic relationship with many other reef inhabitants who benefit from their 'services'. These shrimp wait outside their host and advertise

P. yucatanicus engaged in a commensal relationship with C. gigantae on the drowned cayes belize.

their presence by waving their antennae or swimming up and down in the water column. This serves to bring animals to their location, so that the shrimp can remove and ingest dead skin cells, fungus, and parasites. Often a 'cleaning station' is rather apparent, with several, often large fish waiting their turn to be cleaned. Overall, information regarding this genus is scarce. Information regarding the species that *Periclimenes* spp. inhabits are as follows:

Entacmaea quadricolor, or the bubble-tipped anemone, is a common anemone, as it is found in shallow coastal waters from Central America to Japan. There are several color variations, ranging from a cream color to a vivid red. *E. quadricolor* can attain sizes of up to ~25 cm, though it is difficult to measure the exact and maximum size of an anemone. This anemone is also a common host for the Maroon Clownfish, *Premnas biaculeatus.* This species of anemone is commonly kept by the home aquarist, and is one of the easier species to maintain.

Condylactys anemone forcibly creating a home for itself among an unknown species of scleractinian coral on the belize barrier reef.

Condylactys gigantae, or the Giant Caribbean Anemone, is another very common anemone, with a range extending from Cozumel to Bermuda, and any shallow coastal waters in between. *C. gigantae* attains a maximum size of ~30 cm, and is a common host for many species of the genus *Periclimenes*. This anemone species is very easy for the home aquarist to maintain, as it can adapt to a wide range of lighting and tolerates less than optimal water conditions.

Bartholomea annulata, or the Corkscrew anemone, is found from the Florida coast south to Brazil. A common host for snapping shrimp, as well as *Periclimenes* spp. *B. annulata* attains a maximum size of approximately 15 cm.

Aiptasia pallida is found throughout the shallows waters of the Atlantic, and members of the genus are found on reefs worldwide. *A. pallida* is small compared to the other host anemones for *Periclimenes*, attaining a maximum size of 5 cm. However, this anemone has the most potent nematocysts of any species listed, capable of quickly paralyzing fish many times larger than itself. This is considered a pest anemone species by aquarists, and should not be introduced if possible.

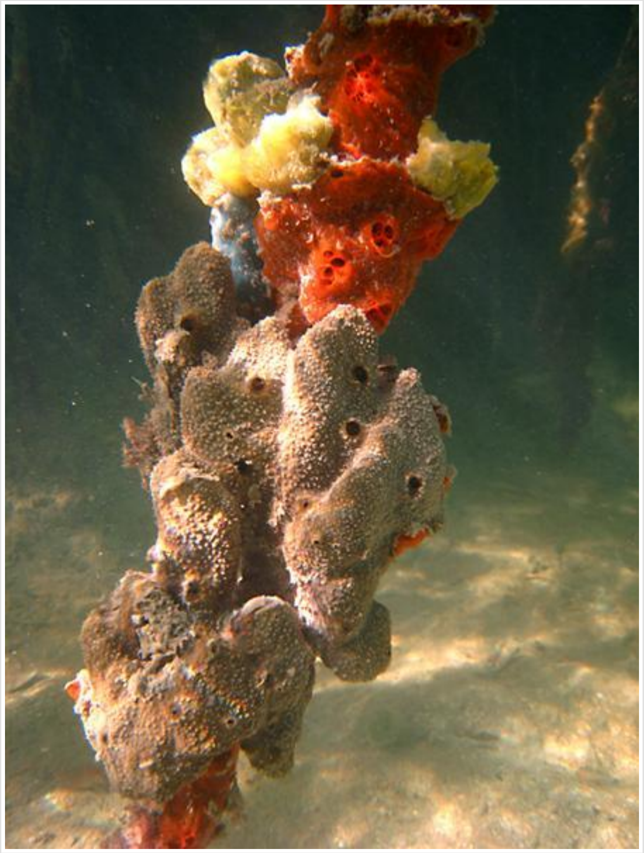
Stichodactyla helianthus, or the Caribbean Carpet Anemone, is a large 'carpet' anemone, with short tentacles and a strong sting. This species is common along the East coast of Central and South America. It is host to the banded crab (*Mithrax cinctimanus)* as well as *Periclimenes* spp. This species is sometimes available under "assorted carpet anemone" or similar, and is a fairly hardy carpet anemone.

Other symbiotic relationships I observed were *Elacatinus oceanops,* the Neon Goby, alone and in pairs on several large pieces of brain coral. The neon gobies were often engaged in the 'cleaning' of other fish species. Large jacks, groupers, and angelfish were usually swimming nearby, waiting for their turn to have parasites removed by this diminutive fish. *E. oceanops* resides in the Western Central Atlantic, ranging from Southern Florida to Belize.[3] E. oceanops and other species of its genus are well knows for their 'cleaner stations' they run on prominent areas of the reef. Their services are used by many species, including fish, turtles, rays, and octopus, some of which would normally prey on gobies and similar fishes. (Johnson & Chase, 1982) I also observed *Remora remora*, the common Remora, engaged in a phoresis relationship with *Sparisoma viride*, the Stoplight Parrotfish on multiple occasions. *R. remora* is found in tropical coastal waters virtually worldwide, and *S. viride* ranges from the east coast of Florida, and south to the Brazilian coast. I often observed multiple remoras attached to or swimming with stoplight parrotfish. Young Remoras can be beneficial to their host, as they remove and ingest parasites, and adult remoras simply participate in phoresis. Remoras commonly scavenge leftovers from their host's meal, and may also ingest feces from the host. They are known to attach themselves to manta rays, whale sharks, turtles, swordfish, tuna, ocean sunfish, and many other large species.

Neon goby perching on a cleaning station on the belize barrier reef.

P. yucatanicus engaged in a commensal relationship with C. gigantae in the drowned cayes belize.

There are many other symbiotic relationships occurring in the Caribbean that I did not personally observe. Amphipods of the genus *Leucothoe* are known to live commensally in sponges, tunicates, and bivalves worldwide. (Thomas & Klebba, 2006) These amphipods spend their entire lives inside their host, relying on the host for protection, as well as for nutrition (which is derived from the water intake of the host). Often in the case of sponges and tunicates the host will completely grow over and encase the amphipod, with no apparent adverse effects – in fact, this may be beneficial in preventing predation to the amphipod.



Sponges and tunicates encrusting a mangrove root in the drowned cayes belize.

Stichodactyla spp. on a rock crevice near the belize barrier reef.

A very common and not always apparent mutualistic relationship is the one between zooxanthellae alga and the tissues of animals, most notably Anthazoans. These dinoflagellates species of the genus *Symbiodinium* inhabit the tissues of a vast majority of corals and anemone species, as well as jellyfish, clams, nudibranchs, and radiolarians. Zooxanthellae are autotrophic, and provide carbon fixation for their hosts, generating up to 90% of their hosts' energy requirements. (Rudman, 2000. Sumich, 1996) In return, the Zooxanthellae receive shelter, protection, access to light, and a constant supply of carbon dioxide, as well as other metabolic wastes. This mutualistic symbiosis has enabled modern corals to be amazingly successful since the mid-Triassic, allowing hermatypic coral species to generate immense amount of physical reef structure from their skeletons, and creating incredibly large reef ecosystems in coastal, tropical waters worldwide. (Ezaki, 1990) These coral reefs have allowed millions of other species to evolve and flourish, and develop symbiotic relationships of their own, in a habitat that simply would not exist without this mutualistic relationship.

Symbiotic relationships are incredibly diverse, and also incredibly common. Many of the species that exist today would never have evolved without symbiotic relationships, both marine and terrestrial. Researching these relationships helps to illustrate how important life is to other life, and what an incredibly complex ecosystem Earth supports, and that ecosystem should not be taken for granted.

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LATERAL LINES

PROBIOTIC ANALYSIS PART I: AEROBIC AND ANAEROBIC CULTURES

By Adam Blundell MS, Robert Durrant

Aquatic & Terrestrial Research Team. While Probiotics continue to grow in popularity, it is important to look at the actual organisms found in culture.

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With the increased usage in Probiotic foods for the aquaculture industry, there is a growing interest from hobbyists. If these foods are increasing the survivability of larval animals in open pens, why not in controlled aquariums? Also, these foods are being used to raise crustaceans as well as fishes. Could this be applied to corals and other reef tank inhabitants?

The usage of probiotics comes in a few forms. First of all some foods are designed to be used by the brood stock. The idea-produce healthy adults; get healthy eggs, grow healthy babies. Be it juvenile fishes, larval shrimp, or healthy medusa. Another method for using probiotic foods is to feed them directly to the gut of first stage animals. In this case the probiotic is the food source, or is added to a food source to be consumed the by the "baby animal" we are trying to raise. A third method is to produce a probiotic environment. In this case the aquarium is dosed with probiotics and the water is kept with a certain level of these biotics at all times.

UPCOMING PROJECTS

We're working with hobbyists in different parts of the nation to test out some of the probiotic foods available to the hobby. Early tests include Cleaner Shrimp, Seahorses, Banggai Cardinals, and Clownfishes. All animals which have been heavily tested, and with some to great success. Another area of testing is in regards to coral fragging. It has been hypothesized that by treating corals with probiotics prior to, or possibly just after, fragmentation could lead to reduced infection and increased survivability. One of the real issues encountered by researchers in this field is that showing positive effects of probiotics is very difficult. Like the testing of many aspects of nutrition and aquaculture, it is easy to show what doesn't work, and difficult to show what does. Upcoming articles will compare the success rate of growing a "batch" of fishes (say Seahorses) in similar setups but with varying levels of probiotics in their foods.

In order to really test a range of probiotics we first need to identify the biotics being used and tested. This first project is designed to let us know exactly with what biotics we are testing.

INTRODUCTION

With the increased development of probiotics in laboratory settings, we are seeing an increase in probiotic foods. Commercialization of probiotics is gaining strength in the human foods production, as well as in aquaculture. Increased production in aquaculture has been linked to probiotic foods, with claims that these foods contain *Bacillus* spp., a gram positive bacterium. This project looked at one of the major sources of aquaculture probiotics, and analyzed the bacteria used in the substance.

IDENTIFICATION OF BACTERIA (AEROBIC)

Currently shrimp farms are testing (with very positive results) the usage of INVE Technologies Sanolife line of probiotics. If you aren't familiar with INVE they are a large commercial aquaculture company with offices around the world (based out of Belgium). INVE has developed a food source, which they claim is inoculated with millions upon millions of living bacteria. They claim that the bacteria are *Bacillus subtilis, Bacillus licheniformis, and Bacillus pumilu*.

HYPOTHESIS

We expect to recover three distinct *Bacillus* isolates. *Bacillus* is a genus of gram positive bacteria that can be easily recognized. These cultures should grow very well in aerobic conditions, but not as well in anaerobic conditions.

However, given the importance of anaerobes in digestion, we would not be surprised to see anaerobes in the mixture. Determining whether or not anaerobes are present in this mixture is also a core purpose in this study.

AEROBIC TESTING PROTOCOL

We started with a sample of Sanolife MIC and cultured the material for bacteria. The original mixture for germination was prepared following the instructions given on the INVE packaging. Bacterial culture was performed using standard laboratory practices, which we have provided here.

Materials:

- Probiotic (Sanolife MIC)
- 0.85% sterile deionized water
- Colorimeter
- 50 ml conical tube
- 15 ml conical tube (2)
- Snap-cap tube
- 0.001 ml calibrated loop
- Sterile transfer pipette
- Fastidious Broth (FB) (2)
- Sheep Blood agar plate (SB) (4)
- Chocolate agar plate (CHOC) (2)
- Colistin-Nalidixic Acid agar plate (CNA) (2)
- MacConkey agar plate (MAC) (2)
- Brucella agar plate (BRUC) (2)
- Bacteroides Bile Esculin/Laked-Blood Kanamycin Vancomycin bi-plate agar (BBE/LKV) (2)
- Phenylethylalcohol agar plate (PEA) (2)

Procedure:

1. Aliquot 50ml sterile deionized water to 50ml conical tube
2. Weigh 0.1g Sanolife in a sterile weigh boat
3. Add 0.1g Sanolife to 50ml sterile deionized water
4. Vortex well
5. Allow solution to sit for 1 hour
6. Vortex well
7. Aliquot 2ml of solution to sterile snap-cap tube
8. Read turbidity of solution using a colorimeter
9. Adjust solution to 0.5 McFarland using sterile deionized water as needed
10. Add .005ml of solution to a 15 ml sterile conical tube containing 5 ml of sterile deionized water to create a 1 x 10^5 CFU/ml solution. This is dilution #1.
11. Vortex well
12. Take 0.5 ml of dilution #1 and add to a 15 ml sterile conical tube containing 4.5 ml of sterile deionized water to create a 1 x 10^4 CFU/ml solution. This is dilution #2.
13. Plate dilution #1 to a SB plate using a .001 ml calibrated loop, streaking quantitatively. Incubate in CO2.
14. Repeat step #13 for dilution #2.
15. Plate dilution #1 and dilution #2 to remaining culture media using one drop per plate. Streak plates for isolation. Incubate media as dictated by type.
16. Read cultures @ 24 hours; perform subculture and identification as necessary.

Reference:

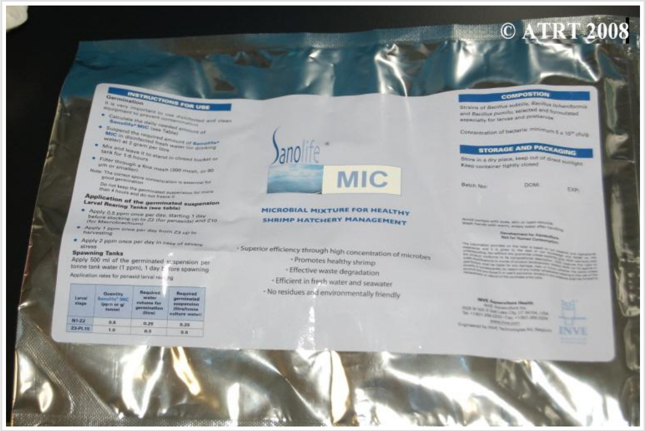
- Original concentration of bacteria: minimum 5 x 10^{10} CFU/g *per package insert*
- Strains of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilu* **per package insert*
- 0.5 McFarland ≈ 1 x 10^8 CFU/ml
- Dilution #1 ≈ 1 x 10^5 CFU/ml
- Dilution #2 ≈ 1 x 10^4 CFU/ml
- 1 colony = 1000 CFU/ml when using a .001 ml calibrated loop

CULTURE RESULTS

Seven types of solid media and one liquid medium were used to culture the bacteria. Our initial hypothesis was to verify the presence of the probiotics, as well as to locate any anaerobes which we do not expect to find in the sample.

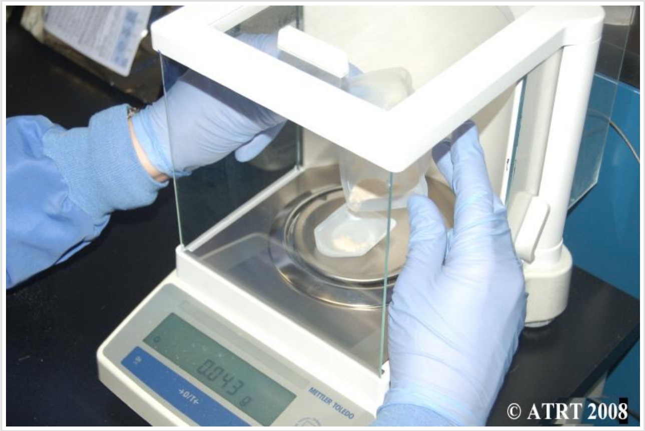
Author Robert Durrant sterilizing the measuring equipment.

Author Robert Durrant preparing original solution.

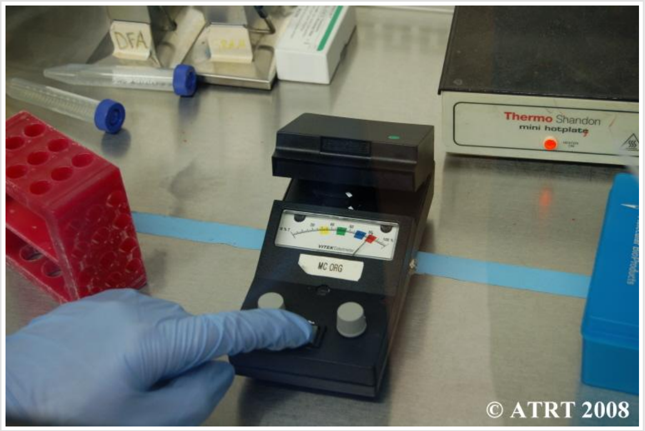


Package of Sanolife probiotic.

Cabinet with standard microbiology agar plates.



Author Robert Durrant weighing out sample.



Author Robert Durrant using a colorimeter to measure turbidity.

Author Robert Durrant streaking a plate quantitatively.

Author Adam Blundell staining slides.

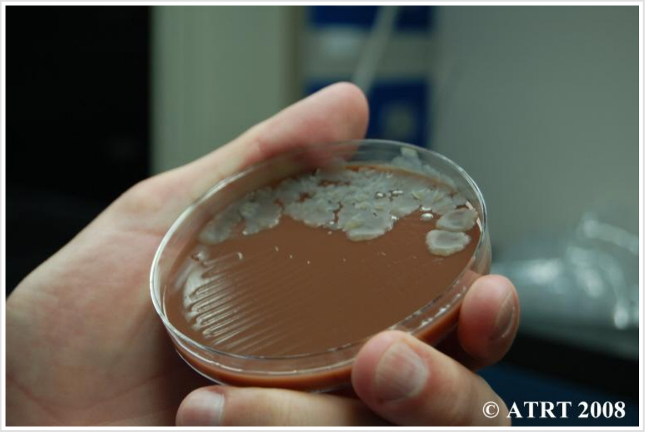
Growth comparison of 24 hour old fastidious broths with a clear solution.

The Key Findings:

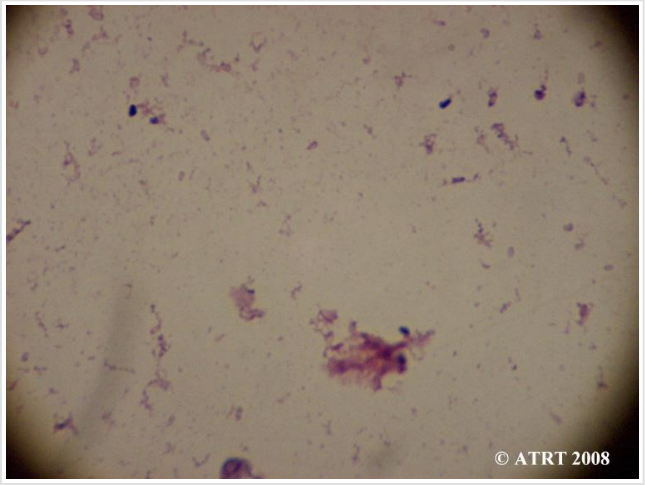
1. The culture did not contain gram negative bacteria.
2. The culture did in fact contain three species of *Bacillus*.
3. *Bacillus* organisms were not fastidious but did grow much better on the chocolate agar.
4. The *Bacillus* isolates grew under anaerobic conditions, but no obligate anaerobes were present.

FINAL CONCLUSION

While Probiotics continue to grow in popularity, it is important to look at the actual organisms found in culture. There is indeed a varied mixture of probiotics and in the case of Sanolife MIC



Mixed 24 hour growth on a chocolate agar plate.



A mixture spore-forming gram positive rods are seen in this microscopic photo of a stained slide prepared during this study.

the organisms are three different strains of *Bacillus* spp. Our studies show this source to be active, and without contaminants.

CONTACT

For questions and comments about this article and the related upcoming projects please contact the authors at adamblundell@hotmail.com.

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